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Receptor-Mediated Neurotransmission in the Hippocampus

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We have been testing the hypothesis that chronic alcohol exposure alters expression and/or function of kainate receptors (KA-Rs) in the hippocampus. KA-Rs control hippocampal excitability and an ethanol-induced upregulation of KA-Rs could contribute to the hyperexcitability associated with alcohol withdrawal. Unexpectedly, Western immunoblotting and immunohistochemical studies have demonstrated that chronic ethanol exposure does not upregulate KA-R subunit expression. However, studies with cultured neurons suggest that alcohol withdrawal itself, but not chronic ethanol exposure, upregulates KA-R function. Therefore, we are now focusing our efforts at testing if this is also true for animals exposed to ethanol. We have switched to a different method of alcohol exposure; we are using the inhalation route, which reproducibly produces high ethanol levels. We are currently processing brain sections from control rats and rats withdrawn from a 14-day inhalational ethanol exposure paradigm. These sections will be immunostained with anti-GluR6/7 antibodies and will be used for radioligand binding experiments. We have also characterized the acute effects of ethanol on the function of interneuronal KA-Rs. Moreover, a preliminary experiment with hippocampal slices from rats withdrawn from a 6-day inhalational exposure paradigm suggests that the function of these receptors is upregulated. During the last year of support, we will concentrate on characterizing the function of KA-Rs in the CA1 and CA3 regions in alcohol withdrawn rats.

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INTRODUCTION:

Alcohol-related medical disorders affect many organs and systems of the body, including the central nervous system (CNS). As with other drugs of abuse, long-term alcohol ingestion results in the development of tolerance, addiction, and dependence. Alcohol produces these effects by altering the actions of neurotransmitters and their receptors in the brain. Chronic ethanol exposure has complex and long-lasting effects on the function and/or expression of a myriad of neurotransmitter receptors and their modulators. A group of proteins affected by chronic ethanol exposure are ligand-gated ion channels such as the glutamatergic ionotropic receptors. Glutamate activates three major classes of ionotropic receptors. These three major types of channels are the NMDA, AMPA and kainate receptors (KA-Rs). The purpose of this proposal is to test whether or not chronic ethanol exposure results in alterations in subunit expression and/or function of KA-Rs in the hippocampus. Maladaptive changes in hippocampal KA-R expression could contribute to the pathophysiology of alcohol withdrawal syndrome. The alcohol withdrawal syndrome is associated with neuronal hyperexcitability and seizures. Since kainate receptors are important regulators of excitability in the hippocampus, upregulation of these receptors may have an important role in the pathophysiology of alcohol withdrawal syndrome.

BODY:

Our overall strategy is to perform studies that assess KA-R expression and function in parallel. Western blot, radioligand binding and immunohistochemical experiments are being used to determine the effects of chronic ethanol exposure and withdrawal on the expression levels of these receptors. Patch-clamp electrophysiological experiments with hippocampal slices are being used to determine the functional consequences of chronic exposure to ethanol and withdrawal. Our specific objectives are:

Objective #1: To determine whether chronic ethanol exposure results in a change in expression of kainate receptors. We are measuring [^3H] vinylidene-kainate binding to hippocampal tissue sections from control, chronically ethanol-treated rats and ethanol-withdrawn rats. We are also assessing specific changes in kainate receptor subunit expression. Specifically, we have measured levels of GluR5, GluR6/7 and KA2 subunits in hippocampal homogenates by using Western blot techniques and will be also assessing KA-R subunit levels in hippocampal tissue sections by using immunohistochemical techniques. These immunohistochemical experiments will determine specific hippocampal neuronal populations where kainate receptors are affected by chronic ethanol exposure and/or withdrawal.

Objective #2: To determine whether chronic ethanol exposure results in changes in the function of pre- and postsynaptic kainate receptors in rat hippocampal CA1 and CA3 pyramidal neurons. We are using whole cell patch-clamp electrophysiological methods to determine the effects of chronic ethanol exposure and withdrawal on synaptic and agonist-evoked kainate currents. We are also measuring effects on presynaptic kainate receptor-mediated inhibition of evoked excitatory and inhibitory synaptic currents in rat hippocampal CA1 and CA3 pyramidal neurons.

The following **Statement of Work** was proposed to complete these objectives:

Year #1:

We will perform Western blot and immunohistochemistry experiments, quantify, and interpret the results of these experiments. We estimate that we will be able to complete experiments with anti-GluR6/7 antibodies during the first year. We will also initiate the electrophysiological characterization of kainate receptor-mediated synaptic and evoked currents in the CA3 region of the hippocampus. We will present our preliminary findings at a scientific meeting.

Year #2:

We will continue Western blot and immunohistochemistry experiments, quantify, and interpret the results of these experiments. We estimate that we will be able to complete experiments with anti-GluR6/7 antibodies and initiate studies with anti-GluR5 antibodies during the second year. We will complete the electrophysiological characterization of kainate receptor-mediated synaptic and evoked currents in the CA3 region of the hippocampus. We will initiate the electrophysiological characterization of kainate receptor function in the CA1 region of the hippocampus, including experiments on kainate receptor-mediated regulation of GABA release. We will present our preliminary findings at a scientific meeting.

Year #3:

We will finish Western blot and immunohistochemistry experiments, quantify, and interpret the results of these experiments. We will complete experiments with anti-GluR5 antibodies and with anti-KA2 antibodies during the last year. We will also complete electrophysiological experiments of kainate receptor function in the CA1 region of the hippocampus, including experiments on kainate receptor-mediated regulation of GABA release. We will present our preliminary findings at a scientific meeting. We will submit a paper to a peer-review scientific journal reporting the findings of our study.

Research Accomplishments:

Western Blot Studies:

Western blot experiments with for all kainate receptor subunits were completed ahead of schedule in Year #1. Findings of these studies were published in Ferreira et al., Ionotropic glutamate receptor subunit expression in the rat hippocampus: lack of an effect of a long-term ethanol exposure paradigm. *Alcohol Clin Exp Res* 25, 1536-1541, 2001 (see appendix).

Immunohistochemistry and Receptor Autoradiography:

We have completed the analyses of confocal images acquired from brain sections from rats exposed to a liquid diet containing ethanol and pair-fed controls. As for the Western immunoblotting studies, these

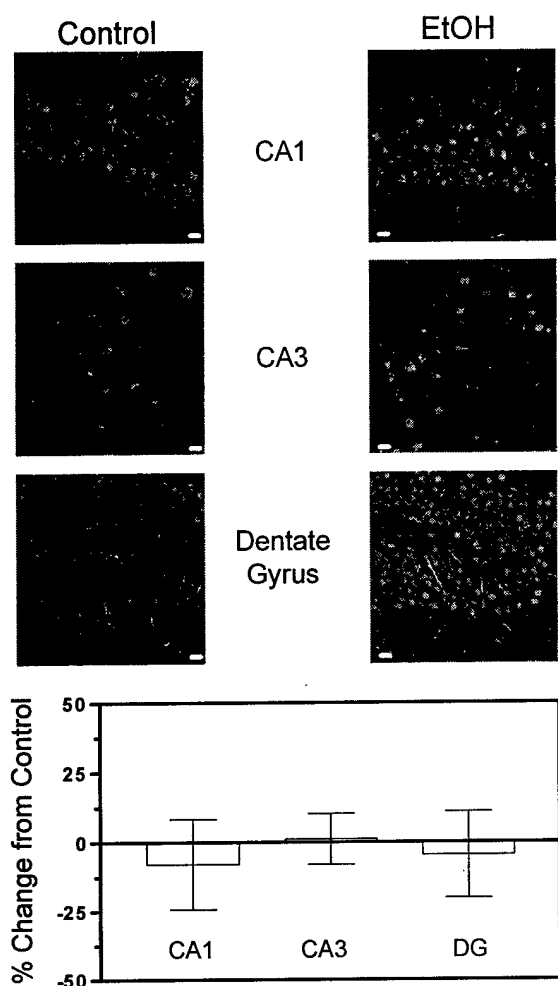


Fig 1. Effect of 16-day EtOH liquid diet on GluR6/7 expression in the hippocampus

ad libitum. Rats were euthanized 24 hours after withdrawal from the 14-day ethanol exposure. Brains from these animals were frozen and sectioned. We are currently processing them for immunohistochemical and binding assays. We hope that, as in the case of the cultured neurons, withdrawal from these higher ethanol exposure levels upregulates kainate receptors in the hippocampus and perhaps other regions of the brains of these rats.

In the previous report, we mentioned that we were in the process of completing autoradiographical studies using [^3H] vinylidene-kainate on the brains of rats exposed to the 16-day liquid diet. Unfortunately, quantification of x-ray films yielded inconclusive results due to high background (Fig 2A-compare with result of a typical assay shown in Fig 2B; note the well-defined band of binding in the CA3 region in panel B). The reason for the high background is unclear at this point but it may have to do with the particular batch of [^3H] vinylidene-kainate used for these studies. We will ensure that new batches of radioligand give optimal results prior to performing further experiments. Given the

brains were obtained from rats euthanized on the last day of the 16-day ethanol diet at the peak of ethanol consumption to prevent ethanol withdrawal. As shown in Fig 1, the chronic ethanol diet did not affect expression levels for GluR6/7 kainate receptor subunits ($n = 10$ animals per treatment group; scale bar = $10\ \mu\text{m}$). These results confirm those of our Western immunoblotting experiments indicating that kainate subunit expression is not upregulated in response to chronic ethanol exposure. These findings are also in agreement with our recent report that chronic ethanol exposure of cultured hippocampal neurons does not upregulate kainate receptor-mediated currents or subunit expression (Carta et al., 2002). However, the experiments with cultured neurons suggest that ethanol withdrawal itself upregulates kainate receptors (Carta et al., 2002). Therefore, we are now focusing our efforts at determining if this effect can also be detected in rats undergoing alcohol withdrawal. We recently acquired the necessary equipment to expose rats to ethanol via the inhalation route. We just completed a exposure paradigm using these inhalation chambers, which allow us to reproducibly induce high blood alcohol levels in the rats. We exposed 8 rats to air plus ethanol vapor and 8 rats to air alone for 6 hours a day for 14 days. Rats achieved peak blood alcohol levels ranging between 0.3-0.4 g/dl (legal intoxication level is 0.08 g/dl); these alcohol levels returned to baseline after 10-12 hrs. Rats received Purina Chow and water

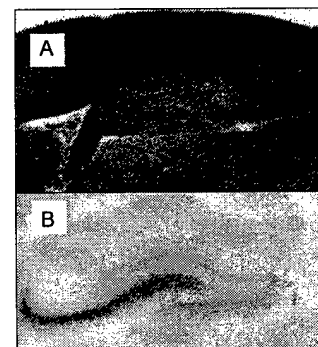


Fig 2. sample of [^3H] vinylidene-kainate autoradiography

negative results obtained with our confocal microscopy studies of the same brains, we have decided not to repeat these studies. As mentioned above, we will instead measure [^3H] vinylidene-kainate binding on brain sections from rats withdrawn from the vapor chamber exposure. We should complete this quantification over the next 2-3 months. Preparation of the brain sections and these receptor autoradiography studies are being performed in the laboratory of the co-investigator, Dr. Savage.

Slice electrophysiological studies:

During Years #1-2, we originally proposed to initiate studies of kainate receptor function in the CA3 region and to initiate experiments in the CA1 region. During the last two years, we have actually focused on characterizing the effects of ethanol on CA1 kainate receptors. Experiments on CA3 kainate receptors will be performed in Year #3. Prior to performing electrophysiological experiments with slices from animals chronically-exposed to ethanol, we had to characterize the acute effects of ethanol on these receptors. We concentrated our efforts on interneuronal kainate receptors, since this population of receptors is the only one that is activated by synaptic release of glutamate in the CA1 region (Cossart et al., 1998; Frerking et al., 1998).

Preliminary data collected in Dr. Weiner's laboratory demonstrated that ethanol potently inhibited kainate-mediated inhibition of GABA_A synaptic responses recorded from CA1 pyramidal neurons in rat brain slices. Although we hypothesized that ethanol was acting to inhibit the function of presynaptic kainate receptors, several conflicting mechanisms had recently been proposed to account for the inhibitory effects of kainate on GABAergic synapses in the hippocampus (Cossart et al., 1998; Frerking et al., 1998; Rodriguez-Moreno and Lerma, 1998). We therefore carried out several additional experiments to clarify the mechanisms underlying the effects of kainate and ethanol under our recording conditions. The results of these studies have been accepted for publication in the Journal of Pharmacology and Experimental Therapeutics (Crowder et al., see appendix). Briefly, we found that kainate inhibition of GABA_A IPSCs was completely blocked by pretreating slices with a GABA_B receptor antagonist, consistent with the results of another study on the effects of kainate receptor activation at these synapses (Frerking et al., 1999). We next determined if ethanol was acting at the level of the presynaptic kainate receptors or downstream on the GABA_B receptors that contributed to the reduction in evoked GABA_A IPSCs. Our results demonstrated that, although ethanol significantly inhibited kainate inhibition of GABA_A IPSCs at concentrations as low as 20 mM, ethanol (80 mM) did not block the inhibitory effect of a GABA_B receptor agonist on GABA_A IPSCs.

More recently, work performed in both the Valenzuela and Weiner laboratories provided more direct evidence of a potent effect of ethanol on interneuronal kainate receptors (see manuscript in appendix by Carta et al). Kainate receptors are important modulators of interneuronal excitability in the CA1 region of the hippocampus. Activation of these receptors increases interneuronal firing, which robustly increases spontaneous inhibitory postsynaptic currents in pyramidal neurons. We found that ethanol potently inhibits this kainate receptor-mediated effect at concentrations that can be achieved in blood after the ingestion of just 1-2 drinks. This effect was due to inhibition of interneuronal firing in response to kainate receptor activation. Thus, ethanol increases excitability of pyramidal neurons indirectly by inhibiting the kainate receptor-dependent drive of GABAergic interneurons. We postulate that this effect may explain some of the paradoxical excitatory actions of this widely abused central nervous system

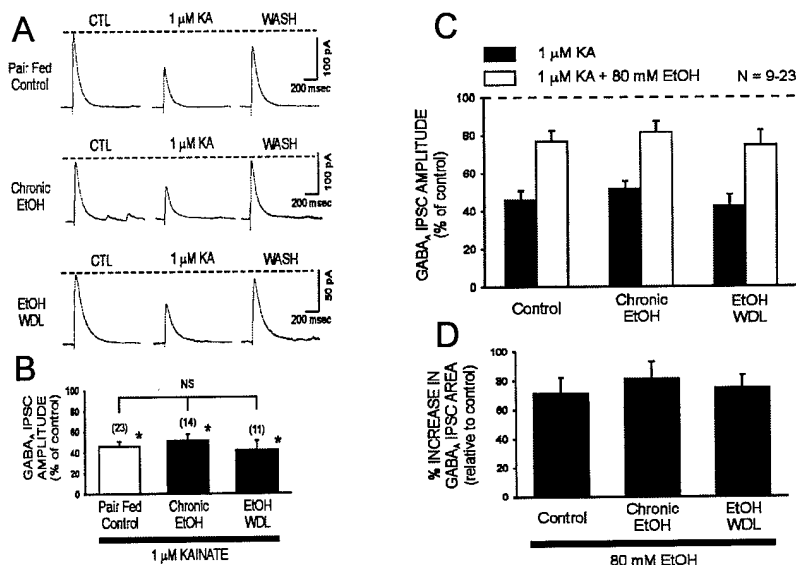


Fig 3. Effects of Chronic EtOH and Withdrawal on KA-Rs at GABA synapses and GABA_A receptor function

ethanol treatment. The results of these studies are summarized in Figs 3A-B. Although rats maintained on the liquid diet achieved blood ethanol levels of approximately 50 mM (~0.25 g/dl), chronic ethanol exposure had no effect on the KA-R-dependent modulation of GABA_A IPSC amplitude (Fig 3A-B). In addition, the acute potentiating effect of ethanol on GABA_A IPSCs was also not affected by this treatment protocol (Fig 3C-D). Moreover, identical results were observed in slices

prepared from rats that had been withdrawn from ethanol for 24 hours (Fig 3A-B). These results suggest that although presynaptic kainate receptors at GABAergic synapses in the rat hippocampus are potently inhibited by acute ethanol exposure, no adaptation to this effect occurs following repeated exposure to ethanol. The fact that the magnitude of these acute synaptic interactions are not diminished following repeated ethanol exposure suggests that these effects may play a significant role in the synaptic mechanisms associated with repeated and excessive ethanol consumption. Alternatively, the liquid diet protocol employed in these studies may not provide a sufficiently high level of ethanol exposure to trigger adaptive changes.

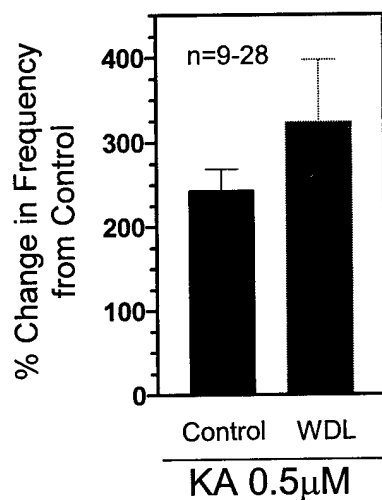


Fig 4. Effect of withdrawal from a 6-day inhalational ethanol exposure on the KA-R-mediated increase in sIPSC frequency in CA1 pyramidal neurons

To test this possibility, a pilot experiment was performed in Dr. Valenzuela's laboratory to evaluate the effect of ethanol withdrawal on interneuronal kainate receptor function (Fig 4). We exposed rats to ethanol vapor for 6 days (blood alcohol level ~0.4 g/dl) and assessed the effect of kainate receptor activation on sIPSC frequency 24 hr after withdrawal from this exposure. We found that there is a trend toward an increase in

depressant. The excitatory actions of EtOH may be perceived as rewarding by some individuals, which could contribute to the development of alcoholism.

Dr. Weiner next resumed studies on the effects of chronic ethanol exposure on interneuronal kainate receptors at GABAergic synapses in the rat hippocampus. Preliminary results completed in Year 1 of this project suggested that our standard 16 day liquid diet protocol had no effect on the function of presynaptic kainate receptors at these synapses. In Year 2, we replicated these initial experiments and also examined the effect of a 24 hour withdrawal following the 16 day

the kainate-induced elevation in sIPSC frequency in slices from alcohol withdrawn rats. These results suggest that interneuronal kainate receptors are upregulated in response to withdrawal from this type of ethanol exposure paradigm. During Year #3, we will repeat this experiment with slices from animals withdrawn from a 14-day inhalational ethanol exposure. We will also directly measure kainate receptor-mediated currents in CA1 interneurons and CA3 pyramidal neurons.

In another related study, Dr. Weiner has continued a project that began last year characterizing the function of kainate receptors in the rat nucleus accumbens. High levels of kainate receptor subunit expression have been reported in this brain region which is thought to contribute to the rewarding effects of ethanol. The results of these studies have resulted in one publication (Crowder et al., 2002) and a second manuscript currently under re-review at the *Journal of Neurophysiology* (Crowder et al., appendix). Briefly, the results of these studies revealed several important roles for kainate receptors in this brain region. Although kainate receptors did not contribute to EPSCs in the principal cells within this brain region (medium spiny neurons), postsynaptic kainate receptors could be evoked by bath application of low concentrations of kainate. We also demonstrated that, as previously observed in the hippocampus, presynaptic kainate receptors were present at glutamatergic synapses within the nucleus accumbens and that activation of these receptors inhibited glutamate release. Interestingly, we also detected presynaptic kainate receptors at GABAergic synapses. Activation of these kainate receptors increased spontaneous and evoked GABA_A IPSCs. In Year 3 of this project, we will characterize the acute effects of ethanol on these newly described populations of kainate receptors as a foundation for future studies investigating neuroadaptation of kainate receptor function in the nucleus accumbens following chronic ethanol exposure and withdrawal.

Plans for Years 3:

We will complete the receptor autoradiographical and immunohistochemical experiments with brain sections from rats withdrawn from ethanol after the 14-day exposure in the inhalation chambers. We will also measure kainate receptor-mediated currents in CA1 interneurons and CA3 pyramidal neurons in hippocampal slices from these rats. If the results of these experiments are negative, we will increase the duration of treatment up to 5-10 weeks. Finally, we will also characterize the acute effects of ethanol on the newly described pre- and postsynaptic kainate receptors in the nucleus accumbens as a foundation for future studies on the effects of chronic ethanol exposure and withdrawal on kainate receptors within this brain region intimately associated with the rewarding effects of ethanol. We will present our findings at a scientific meeting and submit them for publication.

KEY RESEARCH ACCOMPLISHMENTS:

- Quantification of immunostaining with anti-GluR6/7 antibodies in tissue sections from 10 control and 10 rats exposed to ethanol-containing liquid diet. These results confirmed findings of Western immunoblotting studies completed in year 1 that kainate receptor expression is not upregulated by this chronic ethanol exposure paradigm.
- We are concentrating our efforts to determine if kainate receptors are upregulated by ethanol withdrawal itself. Initiated experiments with rats exposed to ethanol via inhalation chambers. This ethanol exposure paradigm yields substantially higher blood alcohol levels than those that can be

achieved with the liquid diet. Exposed 8 rats to air and 8 rats to air + ethanol vapor for 6 hours/day for 14 consecutive days. Rats were euthanized 24 hours after ethanol withdrawal. Froze and sectioned brains with cryostat and are currently performing immunohistochemical and autoradiographical studies on these sections.

- Completed electrophysiological characterization of the effects of acute ethanol exposure on presynaptic KA-Rs at inhibitory GABAergic synapses in CA1 region of the hippocampus. A manuscript reporting these findings is currently in press in *J. Pharmacol. Exp Ther.* Another manuscript has been submitted for publication describing a more detailed characterization of the acute effects of ethanol on interneuronal KA-Rs (Carta et al., see appendix). Completed electrophysiological characterization of the effect of the 16-day liquid diet and withdrawal from this diet (manuscript currently in early stages of preparation). Also completed a pilot experiment with slices from rats withdrawn from a 6-day inhalational ethanol exposure.
- Performed a functional characterization of kainate receptors in the rat nucleus accumbens core region. Results were published in *Journal of Neurophysiology* (see appendix).
- Demonstrated that kainate receptor activation potentiates GABAergic synaptic transmission in the nucleus accumbens core. Revised manuscript currently under review (see appendix).

REPORTABLE OUTCOMES:

Peer-reviewed articles (see appendix):

Ferreira, V.M., Frausto, S. Browning, M.D., Savage, D.D., Morato, G.S., and **Valenzuela, C.F.** Ionotropic Glutamate Receptor Subunit Expression in the Rat Hippocampus: Lack of an Effect of a Chronic Exposure Paradigm. *Alcoholism. Clin. Exp. Res.* 25, 1536-1541, 2001.

Crowder, T.L. and **Weiner, J.L.** Functional characterization of kainate receptors in the rat nucleus accumbens core region. *J. Neurophys* 88, 41-48, 2002.

Crowder, T.L., Ariwodola, O.J., and **Weiner, J.L.** Ethanol antagonizes kainate receptor-mediated inhibition of evoked GABA_A IPSCs in the rat hippocampal CA1 region. *J Pharmacol Exp Ther.* In Press.

Crowder, T.L. and **Weiner, J.L.** Kainate receptor activation potentiates GABAergic synaptic transmission in the nucleus accumbens core. Revised manuscript under review. *J. Neurophysiol.*

Carta, M., Ariwodola, O.J., **Weiner, J.L.** and **Valenzuela C.F.** Alcohol potentially inhibits the kainate receptor-dependent excitatory drive of hippocampal interneurons. Submitted.

Bookchapters:

Valenzuela, C. F. and Savage, D.D. Quantitative analysis of ethanol's effects on neurotransmitter receptor expression. In: *Methods for Alcohol-Related Neuroscience Research*- Liu and Lovinger, Editors. Chapter 5. Pages 113-142. CRC Press. 2002.

Weiner J. L. Electrophysiological Assessment of Synaptic Transmission in Brain Slices.

In: *Methods for Alcohol-Related Neuroscience Research*- Liu and Lovinger, Editors. Chapter 8. Pages 191-218. CRC Press. 2002.

Meeting Presentations:

Ariwodola, O.J., Carta, M., **Valenzuela, C.F.**, and **Weiner, J.L.** Effect of chronic ethanol on presynaptic kainate receptor function at GABAergic synapses in the rat hippocampus. *Alcohol Clin Exp Res* 26 (suppl) 190A: Abstract 625. Presented at Research Society for Alcoholism Meeting, San Francisco, CA, June 28-July 3, 2002.

CONCLUSIONS:

We have established that chronic ethanol exposure via liquid diet does not affect kainate receptor subunit expression. Moreover, the function of kainate receptors in presynaptic terminals is also unaffected by the liquid diet. We are now focusing our attention to establish if alcohol withdrawal itself has an effect, as suggested by experiments with cultured neurons. Initial studies with rats withdrawn from the liquid diet have yielded negative results; kainate receptors in presynaptic GABAergic terminals do not appear to be affected by ethanol. Therefore, we have changed our ethanol administration method to the inhalation route, which reproducibly yields higher blood ethanol levels than the liquid diet. We have extensively characterized the acute effect of ethanol on CA1 interneuronal kainate receptors. We have performed a pilot electrophysiological experiment, which suggests that the function of these interneuronal receptors may be upregulated in slices from rats withdrawn from a 6-day inhalational ethanol exposure. We have processed the brains from rats withdrawn from a 14-day inhalational ethanol exposure for immunohistochemical and binding studies. Functional studies are currently underway to determine if the function of pre- and post-synaptic KA-R is impaired in these animals as well.

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Appendix

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Weiner J. L. Electrophysiological Assessment of Synaptic Transmission in Brain Slices. In: *Methods for Alcohol-Related Neuroscience Research*- Liu and Lovinger, Editors. Chapter 8. Pages 191-218. CRC Press. 2002.

Ferreira, V.M., Frausto, S. Browning, M.D., Savage, D.D., Morato, G.S., and **Valenzuela, C.F.** Ionotropic Glutamate Receptor Subunit Expression in the Rat Hippocampus: Lack of an Effect of a Chronic Exposure Paradigm. *Alcoholism. Clin. Exp. Res.* 25, 1536-1541, 2001.

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Crowder, T.L. and **Weiner, J.L.** Kainate receptor activation potentiates GABAergic synaptic transmission in the nucleus accumbens core. Revised manuscript under review. *J. Neurophysiol.*

Carta, M., Ariwodola, O.J., **Weiner, J.L.** and **Valenzuela C.F.** Alcohol potently inhibits the kainate receptor-dependent excitatory drive of hippocampal interneurons. Submitted.

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5 Quantitative Analysis of Neurotransmitter Receptor Expression with Antibody-Based Techniques

C. Fernando Valenzuela and Daniel D. Savage

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5.1 INTRODUCTION

An important question in alcohol-related neuroscience research is whether ethanol exposure results in maladaptive changes in the expression of neurotransmitter receptors. Alterations in neurotransmitter receptor number, receptor subtypes or receptor subunit composition, appear to have important roles in the development of alcohol tolerance, alcohol dependency and prenatal alcohol-associated changes in neural function. Thus, alcohol researchers are often faced with the need to quantify neurotransmitter receptor expression levels in cultured cells exposed to ethanol, neural tissue from ethanol-treated subjects and also from genetically selected or engineered rodent lines. Quantification of receptor expression in the central nervous system (CNS) is not a trivial task and it usually requires the use of a combination of techniques to ensure the accuracy of results (for instance, see Wu et al., 1995; Petrie et al., 2001). Over the past 20 years, a myriad of laboratory techniques have been used for this purpose, including radioligand binding assays, mRNA quantification methods and antibody-based techniques.

Early studies used radioligand binding to membrane homogenates to assess the effects of ethanol on neurotransmitter receptor levels (Deitrich et al., 1989). Subsequent studies have used receptor autoradiography to localize and quantify, at the histological level, the effects of chronic ethanol exposure on neurotransmitter receptor expression in mature and developing animals (Savage et al., 1991; Negro et al., 1995; Woods et al., 1995; Rothberg et al., 1996; Cowen and Lawrence, 1999; Daubert et al., 1999). To date, receptor autoradiography techniques are still widely used in alcohol-related neuroscience research because of their relative technical ease and the ability to produce quantitatively reliable and reproducible results. In general, the main steps involved in receptor autoradiography are incubation of tissue sections with the radioligand of choice under optimal conditions, removal of unbound ligand by washing, and autoradiographic detection of the bound ligand using highly sensitive films or phosphorimager screens (Chabot et al., 1996; Charon et al., 1998). However, as knowledge of metabotropic receptor subtypes and ionotropic receptor subunit heterogeneity increased over the past 10 years, the development of subtype- or subunit-selective radioligands for more detailed analysis of neurotransmitter receptors has not kept pace, thus limiting the utility of this approach for studies of receptor subtypes and subunits.

In the absence of subunit- and subtype-selective radioligand markers for measuring receptor protein, investigators have relied on measures of messenger RNA (mRNA) to study the effects of ethanol on receptor subtype or subunit expression. This approach has provided some important insights on how ethanol exposure can alter receptor subtype or subunit expression. Currently, the most widely used techniques to quantify mRNA levels are Northern blot analysis, ribonuclease protection assay, and quantitative reverse transcriptase-coupled polymerase chain reaction (RT-

PCR) (Reue, 1998). Northern blot is the RNA counterpart of the Southern blot technique developed for DNA analysis (Southern, 1975) and it involves the electrophoretic separation of RNA species on the basis of size and their transfer onto a blotting membrane. RNA sequences of interest are detected by hybridization to a radiolabeled probe followed by autoradiography or phosphorimaging. Intensities of the bands of interest can be quantified by densitometry or scintillation counting of excised bands (Reue, 1998). Northern blots have been used to quantify GABA_A receptor subunit mRNA levels in rats chronically exposed to ethanol (Keir and Morrow, 1994) and in ethanol-naïve withdrawal-seizure-resistant vs. withdrawal-seizure-prone mouse brains (Montpied et al., 1991). However, it should be emphasized that this technique is generally regarded as semiquantitative because it is not usually possible to completely control RNA transfer and probe hybridization efficiencies (Reue, 1998). Moreover, this technique is not suitable to detect low abundance mRNA species. Low abundance mRNAs can be detected with either ribonuclease protection assays or RT-PCR.

Ribonuclease protection assays involve hybridization in solution of total RNA with a labeled probe followed by ribonuclease digestion of single stranded probe and RNA. Digestion-protected hybrids can then be analyzed by denaturing polyacrylamide gel electrophoresis. Titration reactions with unlabeled RNA sense transcripts allow absolute mRNA quantification in this type of assay (Reue, 1998). For examples on the use of ribonuclease protection assays to measure the effects of long-term ethanol exposure on neurotransmitter receptor expression, see the papers by Ticku and collaborators (Follesa and Ticku, 1995; Hu et al., 1996; Kalluri et al., 1998) and Wilce and collaborators (Hardy et al., 1999).

To quantify smaller amounts of mRNA, the most sensitive technique currently available is quantitative RT-PCR (see Chapter 5 for details on single-cell RT-PCR). This technique involves the production of a single-strand complementary DNA copy of the RNA of interest by using reverse transcriptase followed by amplification of this DNA by PCR. Absolute quantification of PCR products can be achieved by co-amplification of a series of dilutions of standard RNA (competitive RT-PCR) or by monitoring the kinetics of the PCR reaction in real time by using fluorescence-based detection devices (Freeman et al., 1999). RT-PCR techniques have been used to study the effects of chronic ethanol consumption on GABA_A receptor subunit mRNA expression in rat cerebral cortex (Devaud et al., 1995).

It should be emphasized that none of the techniques described above provides histological information on mRNA expression levels. However, quantification of the effects of ethanol on mRNA levels in specific brain regions and neuronal populations can be accomplished by using *in situ* hybridization. This technique involves the hybridization of tagged DNA or RNA probes to the mRNA of interest in tissue sections (Chabot et al., 1996). For example, this technique has been used to quantify, in discrete regions of the rodent brain, the long-term effects of ethanol on GABA_A (Mahmoudi et al., 1997; Petrie et al., 2001) and NMDA receptor subunit expression (Darstein et al., 2000), brain-derived neurotrophic factor mRNA expression (Tapia-Arancibia et al., 2001), calmodulin gene expression (Vizi et al., 2000) and metabotropic glutamate receptor expression (Simonyi et al., 1996). Moreover, this technique has been used to quantify differences in NMDA receptor subunit mRNA expression

in ethanol-withdrawal seizure-prone and seizure-resistant mice (Mason et al., 2001) and also differences in opioid propeptide mRNA expression in the brains of ALKO alcohol and non-alcohol (AA/ANA) rats (Marinelli et al., 2000).

Although mRNA-based techniques can provide important information on the effects of ethanol on neurotransmitter receptor expression, it must be kept in mind that results obtained with these techniques must be interpreted carefully, because changes in mRNA expression are not always predictive of corresponding changes in receptor protein expression or function. Therefore, it is of paramount importance to also measure protein expression levels when performing this type of study. Fortunately, substantial progress in the generation and commercialization of many antibodies for receptor proteins, coupled with major technological improvements in computer-assisted microscopy, densitometry and image analysis techniques has made it increasingly possible to assess ethanol-induced effects on neurotransmitter receptor protein levels using immunoblotting and immunohistochemical techniques. For example, immunoblotting assays have been used to determine the effects of long-term ethanol exposure on the expression of GABA_A receptor subunits (Mehta and Ticku, 1999), ionotropic glutamate receptor subunits (Trevisan et al., 1994; Follesa and Ticku, 1996; Kumari and Ticku, 1998; Chandler et al., 1999) and the effects of fetal ethanol exposure on ionotropic receptor subunits (Costa et al., 2000). Immunofluorescence techniques have been used to assess the effect of long-term ethanol exposure on glycine receptor expression in cultured spinal neurons (van Zundert et al., 2000). However, studies on the effects of ethanol on the expression levels of other neurotransmitter receptors remain to be performed.

In the next sections, we provide a brief description of methodologies that can be used to chronically expose culture cells or animals to ethanol. We then provide a general discussion on the generation and use of different types of antibodies and the application of immunoblotting and immunohistochemical techniques to quantify the effects of long-term ethanol exposure on neurotransmitter receptor protein levels.

5.2 LONG-TERM EXPOSURE TO ETHANOL OF CULTURED CELLS AND ANIMALS

Cultured cells can be useful to study the effects of long-term ethanol exposure on neurotransmitter receptor levels. For example, fibroblast-like cells stably expressing GABA_A receptors subunits (Harris et al., 1998), clonal neural cells (Messing et al., 1986), neuroblastoma-glioma cells (Charness et al., 1986) and primary neuronal cultures from different CNS regions have all been used for this purpose (Iorio et al., 1992; Heaton et al., 1994; Hoffman et al., 1995; van Zundert et al., 2000). In principle, exposure of these cells to ethanol can be easily accomplished by adding ethanol to the culture media. However, caution must be exercised to prevent or compensate for evaporative loss of ethanol, which can be significant at 37°C (Eysseric et al., 1997). For example, this can be achieved by allowing the culture media to pre-equilibrate to the incubator atmosphere, adding the desired

ethanol concentration, and then placing the culture dishes or plates in sealed plastic bags containing a beaker with water plus the same ethanol concentration that was added to the media (Harris et al., 1998). To further compensate for evaporative loss, 50% of the ethanol concentration added the first day of treatment can be added to the culture media every subsequent day (Iorio et al., 1992). For a discussion of alternative methods to chronically expose cultured cells to ethanol, see Eysseric et al., 1997.

The long-term consequences of ethanol exposure can be also studied in animals such as rats, mice, or guinea pigs. A number of methods can be used to chronically administer ethanol to these animals. The most commonly used methods to achieve appreciable blood alcohol levels are the liquid diet technique, the vapor chamber technique and the intragastric intubation technique. In the liquid diet technique, rats are given nothing to eat or drink but an ethanol-containing formula that is introduced progressively (Lieber et al., 1989). This diet can produce blood alcohol levels of 0.08–0.15 g/dl (Lieber et al., 1989; Allan et al., 1998) or even higher (0.2–0.3 g/dl; Trevisan et al., 1994). Controls are pair-fed with an isocaloric formula without ethanol. To determine if the liquid diet has an effect on its own, a control group receiving solid food ad libitum should also be included in the experimental design. The liquid diet technique has been used extensively to study the long-term effects of ethanol on neurotransmitter receptor expression in adult and fetal animals (for example, see Frye et al., 1981 and Costa et al., 2000).

An alternative method to deliver ethanol to animals is the vapor chamber technique (Rogers et al., 1979). In this method, animals are placed in a sealed vapor inhalation chamber and exposed to ethanol vapor in combination with air. Control rats are maintained in the same type of chambers but are exposed to air only. This method can produce blood alcohol levels as high as 0.2–0.3 g/dl (Gruol et al., 1998; Nelson et al., 1999), and it has been widely used to study the chronic effects of ethanol exposure on neurotransmitter receptors in developing and adult animals (Gruol et al., 1998; Jarvis and Becker, 1998; Nelson et al., 1999).

Ethanol can also be administered to animals by gastric intubation, but it must be kept in mind that blood alcohol levels cannot be sustained throughout the day with this method unless the animals are intubated multiple times (Lieber et al., 1989). Relatively short-term treatments (three daily intubations for 6 d) have been successfully used by Ticku and collaborators to study the effects of long-term ethanol exposure on NMDA receptor subunit expression in the rat brain (for example see Kalluri et al., 1998). It is noteworthy, however, that longer treatments are also feasible. Bailey et al. (2001) administered ethanol to pregnant guinea pigs throughout gestation (two daily intubations between gestational days 2 and 67) to assess its effects on GABA_A receptor subunit expression in the cerebral cortex. In this study, pregnant guinea pigs received 4 g/kg of ethanol per day divided into two equal doses given 2 h apart. This treatment resulted in peak blood alcohol levels of ~0.3 g/dl (Bailey et al., 2001). Control animals were gavaged with an isocaloric solution without ethanol.

For a more detailed discussion of the advantages, disadvantages and applications of each of the ethanol administration methods discussed above, the reader is referred to Rogers et al. (1979); Lieber and DeCarli (1982); Lieber et al. (1989).

5.3 GENERATION AND USE OF DIFFERENT TYPES OF ANTIBODIES

The first step in the generation of antibodies is the preparation of the antigen for immunization of the appropriate animal. Antigens of the highest possible purity should be used to obtain antibodies with high specificity. If the antigen DNA sequence is known, then synthetic peptides or bacterially expressed proteins can be used to immunize animals. Synthetic peptides coupled to a carrier protein usually elicit a good immune response. However, antibodies raised against these peptides do not always recognize the native protein. Antibodies raised against bacterially expressed proteins may yield better results if this problem is encountered.

Once the antigen is ready, the appropriate animal for immunization must be chosen. The choice of animal depends on many factors including how much serum is needed and what type of antibody is going to be produced (Harlow and Lane, 1988). For instance, if a large amount of polyclonal antibodies is required, then rabbits or goats should be used. On the other hand, mice are the species of choice for the generation of monoclonal antibodies. Immunization of the animals usually requires the use of an adjuvant to nonspecifically stimulate the immune response. A commonly used adjuvant is the Freund's adjuvant that is a water-in-oil emulsion containing killed *Mycobacterium Tuberculosis* (complete Freund's adjuvant). The first injection of antigen is usually done with complete Freund's adjuvant. Subsequent injections (boosts) are usually performed with incomplete Freund's adjuvant (i.e., without *M. Tuberculosis*). The production of antibodies is checked by obtaining serum samples 7–14 d after a boost injection. Antibody titers are usually checked by radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), or Western immunoblotting. If polyclonal antibodies are being generated, animals are repeatedly boosted and bled at appropriate intervals to obtain sera for antibody purification once a good titer of antibodies has been developed (Harlow and Lane, 1988).

If monoclonal antibodies are being generated, spleen cells from the immunized mice are fused to myeloma cells to produce hybridomas, which are screened for antibody production and those that are positive are expanded and single-cell cloned. Hybridomas can be maintained in a wide range of culture media and readily stored in liquid nitrogen. Monoclonal antibodies can be obtained from tissue culture supernatants or as ascitic fluid. If required, monoclonal antibodies can be further purified as described elsewhere (Harlow and Lane, 1988). Purified antibodies should be stored at relatively high concentrations (>1 mg/ml) in aliquots at -20°C in an isotonic solution such as phosphate buffered saline (PBS) at neutral pH. More diluted antibody solutions should be stored in PBS plus 1% bovine serum albumin (Harlow and Lane, 1988).

It is noteworthy that polyclonal and monoclonal antibodies each have their advantages and disadvantages that should be kept in mind before using them to quantify neurotransmitter receptor expression by Western immunoblotting or immunohistochemical techniques (Harlow and Lane, 1988). In general, most polyclonal antibodies usually yield a stronger signal than monoclonal antibodies in this type of assays. Moreover, most polyclonal antibodies recognize denatured antigens, whereas many monoclonal antibodies do not. However, monoclonal

antibodies usually yield lower background signals and have greater specificity than most polyclonal antibodies. Importantly, a major advantage of monoclonal antibodies is their virtually unlimited supply. In contrast, polyclonal antibody production is limited to the lifetime of the source animal.

For a more detailed discussion on the production, purification, storage, uses and the advantages and disadvantages of monoclonal and polyclonal antibodies, the reader is referred elsewhere (Harlow and Lane, 1988).

5.4 QUANTITATIVE IMMUNOBLOTTING

5.4.1 BACKGROUND

In 1975, Southern developed a blotting technique (Southern blot) to immobilize DNA onto nitrocellulose membranes for further analysis by *in situ* hybridization (Southern, 1975). Others then applied this technique to the study of RNA by *in situ* hybridization (Northern blot) and proteins (Western blot or immunoblot). Immunoblotting is a versatile technique that permits the analysis of a protein based on both its size and binding to specific antibodies (Towbin et al., 1979). The fact that this technique allows the determination of the molecular weight of the immunoreactive species makes it superior to other types of immunoassays, such as ELISA, RIA or dot-blot assay. Although immunoblotting was originally developed as a qualitative technique, it has since been refined and it is now frequently used for quantitative protein analyses. However, several important factors must be taken into consideration when performing quantitative immunoblotting analyses to obtain interpretable data.

A factor that must always be determined prior to performing a quantitative immunoblotting assay is its dynamic working range. This range corresponds to the set of conditions where immunolabeling increases linearly with respect to increasing concentrations of the protein of interest. An immunoassay performed with protein concentrations that fall below or above this range will not provide quantitative information on the expression levels of this protein. The dynamic working range for a given receptor antigen-antibody interaction is specific and must be determined empirically as described in Section 5.4.2. and Appendix 5.1.

Another important point is that a standard curve must be generated and used to calculate relative units of protein expression. Standard curves are widely used in other types of protein determination assays but are often overlooked in immunoblotting studies. The standard curve must be run in the same gel and transferred under the same set of conditions as the samples of interest, i.e., every gel must include a standard curve. If the number of samples requires that multiple gels be run, the standard curve in each gel should be generated from a single sample kept in aliquots at -70°C . This procedure is necessary to allow comparisons among different gels. In all cases, the immunolabeling of the standard curve bands must increase linearly with increasing sample concentrations. This standard curve can be subsequently analyzed by linear regression analysis, from which relative expression levels of the protein of interest can be straightforwardly determined. Importantly, immunolabeling of the samples must fall within the dynamic working range of the standard curve.

Finally, quantitative immunoblots must include internal controls to ensure adequate sample loading and transfer and also for normalization purposes. Determination of the levels of housekeeping genes is a common practice in Southern and Northern blotting assays and it should also be performed in immunoblotting assays. A commonly used control is the determination of the expression levels of a housekeeping protein that is unaffected by the treatment in question (Liao et al., 2000). Among the housekeeping proteins that are frequently used as internal controls are β -actin and tubulin. Ideally, levels of a housekeeping protein should be measured in the same membrane (i.e., by reprobing). If this is not possible, duplicate membranes can be tested. It should be kept in mind, however, that the use of duplicate membranes does not completely rule out pipetting and transferring errors. Alternatively, normalization can be performed with respect to average intensity values obtained from several prominent bands visualized by Coomassie staining of the blotting membranes.

5.4.2 METHODOLOGY

5.4.2.1 Preparation of the Antigen Sample

An important strength of immunoblotting is that virtually any protein sample can be analyzed by this technique. However, the protein sample must be in a buffer that is compatible with the electrophoresis system (i.e., it should have a nearly neutral pH and salt concentrations below 200 mM) and its protein concentration must not exceed the loading capacity of the gel. Samples for immunoblotting do not require elaborate preparation steps in most cases. For instance, samples can be homogenized by sonication in PBS with protease inhibitors. In some instances, however, it may be necessary to prepare cell membranes or to fractionate the samples by using centrifugation or chromatographic methods. Purification by immunoprecipitation techniques can also be used if an antigen is not particularly abundant. In all cases, samples for the generation of the standard curve and for the determination of the levels of the protein of interest should be prepared under identical conditions. One of the most common methods to prepare samples for immunoblotting is to directly lyse cells or tissues (see Appendix 5.1).

5.4.2.2 Electrophoresis

Samples can be separated by different types of electrophoretic techniques, including discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970), non-denaturing electrophoresis and two-dimensional electrophoresis. We will discuss here only the details of discontinuous SDS-PAGE, as this is the most commonly used type of electrophoresis. Before separating the samples by discontinuous SDS-PAGE, two important issues should be considered. First, the amount of protein to be loaded per lane must be determined empirically for each particular antibody and should never exceed 150 μ g of total protein per lane. A recommended starting point would be to dilute an aliquot of the standard curve sample to a concentration of 1 mg/ml of total protein and to load 5, 10, 15, 20, 25, 30, 35 and 40 μ l of this sample on a gel (i.e., 5-40 μ g/lane). This pilot gel

should then be blotted and analyzed as described below to determine if the dynamic working range of the assay falls within this range of total protein concentrations. Second, some consideration should be given to the type of gel to be used. In general, the lower the percentage of bis-acrylamide in the gel and the thinner the gel (commonly between 1 and 1.5 mm), the easier the transfer will be (Harlow and Lane, 1988). Moreover, high molecular weight proteins are difficult to transfer unless they run at least one third of the distance from the top to the bottom of the gel, which can be achieved with lower percentage bis-acrylamide gels (Harlow and Lane, 1988). For discontinuous SDS-PAGE, precasted gels can be purchased from several commercial suppliers such as BioRad (Hercules, CA), Fisher Scientific (Pittsburgh, PA) and others. Alternatively, gels can be prepared as described in Appendix 5.1.

5.4.2.3 Blotting

Before initiating Western immunoblotting experiments, the appropriate blotting membrane must be chosen. It is recommended that an initial comparison be made of the performance of different types of membranes for a particular antigen-antibody pair. The two types of membranes most commonly used today are nitrocellulose and polyvinylidene difluoride (PVDF). Nitrocellulose has high binding protein capacity ($80 \mu\text{g}/\text{cm}^2$) and nonspecific protein binding to this type of membrane can be easily blocked. It does not require pre-activation. It is commercially available in 0.45 and $0.22 \mu\text{m}$ pore sizes. The smaller pore size nitrocellulose is useful for the transferring of low molecular weight proteins (i.e., $<20 \text{ kDa}$). The transfer buffer used with nitrocellulose membranes must contain methanol, as it increases the binding of denatured proteins to this type of membrane. Two disadvantages encountered when using nitrocellulose membranes are the poor transfer of high molecular weight proteins onto these membranes and their lack of mechanical strength. In contrast, PVDF membranes have higher mechanical strength and binding protein capacity ($160 \mu\text{g}/\text{cm}^2$) than nitrocellulose. However, nonspecific binding can be more difficult to block in PVDF membranes than nitrocellulose membranes. Importantly, PVDF membranes must be wetted in 100% methanol to become activated but transfer buffer must not contain alcohol.

Another important issue to consider is the choice of transfer buffer. A widely used transfer buffer contains 25 mM Tris Base (pH 8.3), 190 mM glycine, and 20% reagent grade methanol. This buffer is appropriate for the transfer of most proteins. In our experience, this buffer is usually adequate for proteins $<130 \text{ kDa}$. For the transfer of higher molecular weight proteins, 0.1 % SDS w/v can be added to the transfer buffer. SDS will improve the elution of high molecular weight proteins but it may reduce binding efficiency to nitrocellulose. The use of PVDF membranes with transfer buffer containing SDS but not methanol may result in improved transfer efficiency of high molecular weight proteins. It should be kept in mind, however, that SDS may precipitate below 10°C and that it may increase the conductivity of the transfer buffer, resulting in increased current and heating. Poorly controlled buffer temperature can affect transfer and can also be a safety hazard. The manufacturer's instructions must be consulted to determine the conditions necessary to appropriately control temperature in a particular immunoblotting apparatus. Transfer

of proteins to membranes is usually achieved by electrophoretic elution. Two types of apparatuses can be used for this purpose: semi-dry or wet (also called tank transfer). Wet immunoblotting apparatuses are currently widely used around the world. A detailed protocol for electrotransferring with this type of set up is provided in the Appendix 5.1. For immunolabeling and detection, these steps can be followed:

1. Non-specific binding to the blotting membranes must be prevented prior to probing them with primary antibodies. A solution commonly used for this purpose is known as Blotto/Tween, which contains 5% w/v nonfat dry milk, 0.2 % w/v Tween-20 and 0.02% w/v sodium azide in Tris-buffered saline (TBS), which is composed of 50 mM Tris (pH 7.5) and 150 mM NaCl. If necessary, the concentrations of nonfat dry milk and Tween-20 can be increased to 10% w/v and 0.4% w/v, respectively. Incubation in Blotto/Tween solution for 1–2 h at room temperature is usually sufficient to prevent nonspecific binding. However, membranes can be incubated overnight at 37°C if shorter incubations at room temperature yield unsatisfactory results. If Blotto/Tween-20 does not work under any of the conditions described above, non-specific binding could be blocked with 3% w/v bovine serum albumin in TBS plus 0.02% w/v sodium azide; 0.2% w/v Tween-20 in TBS plus 0.02% w/v sodium azide; or 10% v/v horse serum in TBS plus 0.02% w/v sodium azide. It is noteworthy that milk and albumin have phosphotyrosine, which may cause high background when probing membranes with anti-phosphotyrosine antibodies (Harlow and Lane, 1988).
2. Remove the membrane from the blocking solution and wash it 2×5 minutes with TBS.
3. Incubate with primary antibody (i.e., specific for the protein of interest) in 3% bovine serum albumin in TBS. Polyclonal antibodies that have been purified by antigen affinity chromatography or monoclonal antibodies that recognize denatured epitopes are the preparations of choice for immunoblotting assays. It is noteworthy, however, that polyclonal antibodies are frequently supplied as crude serum or purified immunoglobulin fractions. These preparations could contain virtually all the repertoire of antibodies from the source animal, which can lead to background problems. Thus, an important control that must be performed when using these preparations of polyclonal antibodies is to preabsorb them with the antigen, which should prevent immunolabeling of the protein of interest. Final antibody concentrations usually range between 1 and 50 $\mu\text{g/ml}$, but optimal dilution levels should be determined empirically in a set of pilot experiments. Moreover, the duration of the incubation must be determined in the same manner. Most antibodies work well when incubated for 12–18 h at 4°C with constant shaking. Other antibodies give optimal results with incubations as short as 1 h at room temperature with constant shaking.
4. Wash 2×10 minutes with TBS plus 0.1% w/v Tween-20 (TBST) followed by 2×10 minutes washes with 3% w/v bovine serum albumin in TBS.

5. Incubate with the appropriate secondary (i.e., labeled) reagent. The optimal concentration of the secondary antibody and the duration of incubation must be determined empirically in preliminary pilot experiments. Detection can be accomplished with a variety of reagents, including ^{125}I -labeled protein A or protein G or ^{125}I -labeled species-specific anti-immunoglobulin antibodies. Another group of secondary reagents includes antibodies coupled to enzymes that produce either chemiluminescence (Thorpe et al., 1989; Huang and Amero, 1997) or color reaction products (Harlow and Lane, 1988). Detection of antibodies by chemiluminescence is perhaps the most widely used method of detection today. Kits for chemiluminescence detection are available from several commercial suppliers (for instance, Roche Molecular Biochemicals, Indianapolis, IN or Pierce, Rockford, IL). Detection of fluorophore-coupled secondary antibodies by phosphorimager analysis has been recently put forward as a reliable method to detect antibodies in immunoblotting assays (Fradelizi et al., 1999). In all cases, a control experiment in the absence of primary antibody must be performed with any of these secondary reagents to determine their background signal.
6. Wash the membrane 4×15 minutes with TBST with constant shaking.
7. Radiolabeled secondary reagents and antibodies coupled to enzymes that produce chemiluminescence can be detected with x-ray film or the appropriate phosphorimager screens. Fluorophore-coupled secondary antibodies can also be detected with a phosphorimager. Immunolabeling with enzyme-labeled secondary antibodies can be detected by the addition of chromogenic substrates. For more details, consult the manufacturer's instructions for each specific secondary reagent.
8. Densitometry of the bands of interest is then performed. X-ray films and membranes exposed to chromogens can be scanned and analyzed by a number of commercially available programs such as Image-Pro® Plus (Media Cybernetics, L.P., Silver Spring, MD) and others. Phosphorimager acquired images are usually analyzed with software provided by the manufacturers of this type of equipment.
9. Reprobe the blot with an antihousekeeping protein antibody. This step could be accomplished in different manners. If the membrane shows only a single band corresponding to the protein of interest, it could be rewetted in TBS and directly reprobed with the antihousekeeping protein antibody. However, the molecular weight of this protein should be significantly different from the molecular weight of the protein of interest. Under some circumstances, it may be possible to probe the membrane with a mixture of antibodies against the protein of interest and the housekeeping protein. If chemiluminescence detection methods are used, horseradish peroxidase antibodies bound to the protein of interest can be inactivated by treatment with hydrogen peroxide prior to reprobing (Liao et al., 2000). Alternatively, membranes can be stripped by incubation at 60°C in TBS plus 2% SDS and 0.1 M 2-mercaptoethanol for 30–60 min. Immunoblotting stripping kits are commercially available from, for example, Chemicon

(Temecula, CA) and Pierce (Rockford, IL). Recipes for other stripping solutions can be found elsewhere (Renart et al., 1996). After membrane stripping, wash extensively with TBS, reblock, reprobe and detect as described above. Another possibility would be to stain the membranes, for example with Coomassie blue, and to normalize values of interest with respect to major bands in each lane.

10. Perform densitometric and statistical analyses.

5.4.3 POTENTIAL RESULTS, ANALYSIS AND INTERPRETATION

Following the steps described above, it is rather straightforward to determine the relative concentration of a protein by immunoblotting analysis. Results of this type of assay may appear the same as those shown in Figure 5.1.

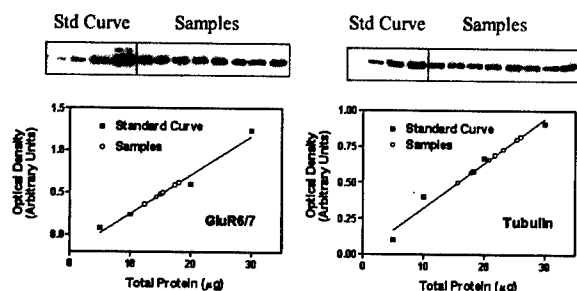


FIGURE 5.1 A quantitative immunoblotting assay for GluR6/7 subunits, which belong to the kainate family of glutamate receptors. It was performed with homogenates prepared by sonication of eight different hippocampi from adult mice. Samples (5–30 μg of total protein per standard curve lane and 15 μg of the unknowns) were separated by discontinuous SDS-PAGE on 7.5 % minigels and transferred onto 0.45 μm pore size nitrocellulose using transfer buffer containing 25 mM Tris Base (pH 8.3), 190 mM glycine, and 20% methanol. Nonspecific binding was blocked with Blotto/Tween-20 for 2 h at room temperature. Polyclonal antibodies against GluR6/7 subunits were purified by antigen affinity chromatography (Upstate Biotechnology, Lake Placid, NY) and were used at a final concentration of $\sim 1 \mu\text{g}/\text{ml}$. Membranes were stripped with a Re-blot Western Blotting Recycling Kit from Chemicon (Temecula, CA) and then reprobed with anti-tubulin monoclonal antibodies from Sigma (St. Louis, MO) at a 1:5,000 dilution. Detection was performed with a chemiluminescence kit from Roche Molecular Biochemicals (Indianapolis, IN). X-ray films were scanned with a Hewlett-Packard ScanJet II scanner, then analyzed with Image-Pro[®] Plus image analysis system (Media Cybernetics, L.P., Silver Spring, MD). Linear regression analysis was performed with GraphPad software (San Diego, CA).

Note that immunolabeling of standard curve samples increases linearly, and immunolabeling of samples of unknown concentrations fall within the range of this standard curve. From this standard curve, relative levels of protein expression can be determined. It should be emphasized that this assay cannot determine the actual protein concentration of the subunit of interest because the standard curve values that are entered in the abscissa correspond to the total protein concentration of the

hippocampal homogenates that were loaded per lane. Therefore, once linear regression analysis and interpolation are performed to determine unknown values, total protein concentrations should be denoted as relative units of protein (GluR6/7 in the example). To determine the actual concentration of a protein of interest, it would be necessary to use a 100% pure preparation of the protein of interest to construct the standard curve.

Also shown in Figure 5.1 is the immunoblotting analysis of a housekeeping protein in the same membranes that had been previously probed with anti-GluR6/7 antibodies. The protein shown in the example is β -tubulin. As can be observed, there is some variability in the immunolabeling of this protein, which suggests that there were small differences in sample preparation or gel loading. In order to correct for these intersample differences, relative units of GluR6/7 protein levels could be divided by the relative units of β -tubulin or a correction factor generated from these tubulin levels.

In the example shown in Figure 5.1, 15-well combs were used, which permit the loading of four samples to construct the standard curve plus eight samples. If one were examining samples from a control and an ethanol-exposed group, this would allow studying just four samples from each group. A sample number of four would be insufficient to reach statistical significance if a small ethanol-induced change in subunit levels is expected. A power calculation performed with StatMate software (GraphPad Inc, San Diego, CA) showed that to obtain a $p < 0.05$ by unpaired t -test with a standard deviation of 20%, the difference in the mean expression levels between samples would have to be ~60% with an $n = 4$. Therefore, it is usually necessary to study larger number of samples, which require analysis by multiple immunoblots. In this case, standard curves should be constructed with a single sample kept in aliquots in a deep freezer to allow comparisons among samples in different membranes.

5.5 QUANTITATIVE IMMUNOHISTOCHEMISTRY

Immunohistochemistry is a widely used technique in neuroscience research. This technique has been mainly used in qualitative studies to detect the presence and localization of neurotransmitter receptors and many other proteins in the nervous system. In the past, immunohistochemistry was not commonly used for quantitative studies. This type of study was generally regarded as difficult because of confounding variables such as variability in sample and antibody preparations, inconsistency in the performance of enzymatic detection systems and microscopes, and lack of appropriate imaging analysis tools (Appel, 1997). In recent years, however, significant progress has been achieved in the generation of reliable secondary immunoreagents and substantial improvements have taken place in the areas of fluorescence and confocal microscopy as well as in the technology for image acquisition and analysis. These technical advances have made it increasingly possible to perform quantitative immunohistochemical studies.

A major advantage of immunohistochemistry over other types of immunoassay is that this technique is performed on tissue with relatively well preserved cytoarchitecture, which permits the evaluation of discrete cell groups and, in some cases,

of specific subcellular compartments. However, a disadvantage of this technique is that the molecular weight of the protein of interest cannot be determined in immunohistochemical experiments. Thus, in contrast to immunoblotting, size cannot be used to identify the specificity of immunolabeling when a particular antibody cross-reacts with more than one protein. Consequently, the use of high quality antibodies is essential for the success of this type of experiment. Moreover, appropriate control experiments, such as the pre-absorption of primary antibodies, must be performed to ensure that immunolabeling is specific for the antigen of interest.

In the next sections, we have elected to review the technical details of quantitative confocal microscope and radioimmunohistochemistry. However, it is important to emphasize that at least one other method has been described that can provide quantitative immunohistochemical information. This method involves analysis of background and stained structures by using the peroxidase-antiperoxidase (PAP) method (Sternberger and Sternberger, 1986).

5.5.1 QUANTITATIVE CONFOCAL MICROSCOPY

5.5.1.1 Background

Because of its high resolution, confocal microscopy has emerged as an important technique to quantify receptor expression in immunolabeled tissues. Optimal illumination, along with the ability to scan samples in all axes, make the laser scanning confocal microscope (LSCM) an ideal tool for measuring receptor levels in immunohistochemical studies. Moreover, imaging illumination, scanning and acquisition parameters are computer controlled, which makes it relatively straightforward to standardize such parameters for the analysis of multiple samples. In addition, images acquired with an LSCM can be subsequently processed with computer software to accurately determine immunofluorescence intensity levels for specific neurotransmitter receptor subunits. Good et al. (1992) were the first to apply confocal microscopy to the quantification of receptor levels in single cells. These investigators demonstrated that epidermal growth factor receptor density could be determined as accurately with confocal microscopy techniques as with ^{125}I -EGF binding techniques. LSCM was subsequently used by Dodge et al. to quantify intracellular levels of a Clara cell secretory 10 kD protein in rat bronchi (Dodge et al., 1994). More recently, Gazzaley et al. used LSCM to quantify the regulation of NMDAR1 subunit protein expression by estradiol in the rat hippocampus (Gazzaley et al., 1996b; Gazzaley et al., 1996a). This technique is currently being used to quantify the long-term effects of ethanol on ionotropic glutamate receptor expression in hippocampal neurons (Valenzuela and Savage, unpublished observations).

5.5.1.2 Methodology

5.5.1.2.1 Tissue Preparation

Sectioning freshly frozen tissues and sectioning tissues prepared from perfused animals are two commonly used methods to prepare samples for quantitative confocal experiments. These two methods will be considered in detail below. It is recommended that a pilot experiment be performed comparing the staining obtained

with tissue prepared by using these two methods to determine which one works best for the antigen-antibody pair of interest. Another method that can be used to prepare samples for quantitative confocal experiments is the sectioning of paraffin-embedded tissues. Although this method has been successfully used in confocal microscopy studies (Nichol et al., 1999), it involves harsh fixation and embedding procedures that could affect the antigen of interest. Therefore, the methods for the preparation of sections from paraffin embedded tissues will not be considered further in this chapter. Details on this technique can be found elsewhere (Harlow and Lane, 1988).

1. For the preparation of frozen tissue sections, begin by rapidly euthanizing animals following the guidelines of the American Veterinary Medical Association (Euthanasia APO, 2001). Remove brains or other tissues of interest rapidly and carefully to prevent damage. Wash by submerging into ice-cold PBS. Wipe off excess PBS with filter paper, gently place it at the end of a spatula bent at a 90-degree angle and submerge it slowly (to prevent cracking) into isopentane that has been chilled in a dry ice-methanol bath. After ~4–5 min (for an adult rat brain), remove brain, wrap loosely in aluminum foil and place into a prelabeled airtight plastic bag. Store immediately at -70°C . Keep on dry ice until transferring to the deep freezer. Frozen samples can be kept in airtight bags at -70°C for several months. Cooling isopentane for freezing whole brains can be achieved by submerging a beaker containing it into a larger container with dry ice-cooled methanol. When submerging the beaker with isopentane, make sure it is covered to prevent drops of methanol from falling into the isopentane. To cool with dry ice, start slowly adding small pieces of it into a suitable container (for instance a Dewar) with methanol that had been pre-cooled overnight at -20°C . The methanol will be ready when adding dry ice pieces causes minimal bubbling. Plan in advance, as cooling the methanol could take about 1–2 hours. Smaller samples than a whole brain could also be directly frozen in liquid nitrogen (Harlow and Lane, 1988).
2. For the preparation of tissue from perfused animals, please first consult with the attending veterinarian at your Institutional Animal Care Facility for guidelines on this type of procedure. Animals should be deeply anesthetized with a suitable agent and then transcardially perfused with cold 1% paraformaldehyde in 0.1 M PBS followed by cold 4% paraformaldehyde in PBS. Brains are then carefully removed and postfixed for 2–3 h with 4% paraformaldehyde in PBS at 4°C . Brains can be stored for several months at 4°C in 5% sucrose in PBS in closed containers to prevent evaporation. To prepare 4% paraformaldehyde, dissolve 8 grams in 100 ml of water, add a few drops of 1 N NaOH, heat at 60°C and stir it in a fume hood until clear. Let this solution cool down to room temperature and mix it with 100 ml of 2X PBS. Paraformaldehyde should be prepared fresh daily.
3. Frozen samples can be sectioned (5–10 μm thickness) with a cryostat following the manufacturer's instructions. Cryostat-prepared sections can be collected on pretreated glass slides such as Superfrost® Plus selected precleaned micro slides (VWR Scientific, West Chester, PA). Alternatively,

clean glass slides can be coated with 1% gelatin (Harlow and Lane, 1988). Sections can be stored in suitable containers kept in airtight bags at -70°C for a few weeks.

4. Once preserved, tissue from perfused animals can be sectioned by freezing as described above or with a vibratome ($40\text{ }\mu\text{m}$ sections). Vibratome prepared sections can be stored in PBS plus 0.1% sodium azide at 4°C .

5.5.1.2.2 Immunolabeling

1. For cryostat prepared sections, thaw them for 3–5 min. Draw a ring around each section with a hydrophobic slide marker specifically designed for staining procedures (also known as a PAP pen; Research Biochemicals, Natick, MA). The purpose of this ring is to hold antibody solutions in place. Incubate sections in fresh 4% paraformaldehyde-PBS (prepared as described above) for 20 min at room temperature. Multiple slides can be incubated simultaneously by using Coplin jars or other commercially available staining dishes (for instance, see Fisher Scientific or VWR Scientific catalogs). Rinse with PBS 3×10 min. Incubation of vibratome-cut sections can be performed in multi-well plates under constant shaking.
2. If the antibody of interest recognizes an intracellular epitope, permeabilize the sections with PBS plus 0.2–0.3% Triton X-100 at room temperature for 20 min. Rinse with PBS 3×10 minutes and dip in water three times.
3. To prevent nonspecific antibody binding, sections are blocked by preincubation (1 h) with PBS (with 0.2–0.3% Triton X-100 if it was used to permeabilize before) plus 2–4% bovine serum albumin or 1–2% purified antibodies from the same species as the detection reagent.
4. Incubate with primary antibody diluted in PBS plus 2–4% BSA (with 0.2–0.3% Triton X-100 if it was used to permeabilize before). Put enough antibody solution to cover the section but make sure it does not overflow the PAP pen ring. Optimal antibody dilutions and incubation times should be determined empirically in a set of preliminary experiments. Whenever possible, polyclonal antibodies purified by antigen chromatography or monoclonal antibodies (purified or in hybridoma supernatants) should be used for this type of experiment. Monoclonal antibodies in ascites fluid, unpurified sera and polyclonal antibodies purified by methods other than antigen chromatography should be used with caution, as these preparations contain nonspecific antibodies from the source animal. Virtually any of these preparations will give nonspecific immunolabeling if used at high enough concentrations. Therefore, these preparations should be used as diluted as possible. Moreover, the specificity of the immunolabeling should be confirmed by incubating dilutions that give optimal results with an excess of the immunizing antigen (i.e., peptide, fusion protein or purified protein). This procedure should result in elimination of the immunolabeling signal coming from the antigen of interest. Another

important control should be to test pre-immune serum. Incubation with the primary antibodies can take as long as 24–48 h and it should preferably be performed in a humidified chamber at 4°C.

5. On another set of sections from the same animal, probe with an antibody that recognizes a housekeeping protein. This procedure will be useful to establish the specificity of changes in the levels of the protein of interest.
6. Gently shake primary antibody solution off the slides. Wash 3×10 minutes with PBS in a Coplin or any other type of jar. Re-apply PAP pen if necessary.
7. Incubate in the dark with a fluorescently labeled secondary immunoreagent, which could be fluorescently labeled protein A or G, species-specific biotinylated antibodies used in conjunction with fluorescently conjugated avidin, or the appropriate species-specific fluorescently labeled secondary antibody. Species-specific antibodies recognize the Fc region of immunoglobulins from a particular species. These immunoreagents are available from several commercial sources, such as Molecular Probes (Eugene, OR), Jackson ImmunoResearch Laboratories (West Grove, PA), and others. Incubation times and optimal dilutions for these secondary immunoreagents should be determined empirically in a set of preliminary experiments. Start by incubating them for 1 h at room temperature in PBS plus 2–4% BSA (with 0.2–0.3% Triton X-100 if it was used to permeabilize before). Importantly, these reagents should be tested in the absence of primary antibodies to determine their background signal contribution. Manufacturers of these reagents often provide recommendations for dilution ranges that can be used as a starting point in histochemical experiments.
8. Gently shake secondary antibody solution off the slides. Wash 3×10 minutes with PBS in Coplin or any other type of jar. Dip three times in water.
9. Add a small drop of mounting media to the specimen. Use of an anti-fading mounting media is recommended (for instance, Fluormount-G from Southern Biotechnology, Birmingham, AL; Slowfade from Molecular Probes, Eugene, OR or AF1 from Citifluor, London, England). Gently, place a coverslip on top avoiding bubbles. Remove excess of mounting medium with a tissue paper. Wait 2 h and then add a small layer of nail polish at the edges of the coverslip. Let the mounting medium set overnight.

5.5.1.2.3 *Image Acquisition with Laser Scanning Confocal Microscope (LSCM)*

1. Specimens from control and treated animals to be analyzed by LSCM should be stained as described above on the same day and under identical experimental conditions. The operator of the LSCM should be blinded to

which sections correspond to which experimental group. At least three non-adjacent sections must be analyzed from each animal.

2. Optimal parameters for confocal imaging acquisition should be established at the beginning of the day; i.e., images from control and treated sample pairs should be acquired under identical conditions. Ideally, these settings should remain constant throughout the project. In some cases, settings must be changed to analyze fields in different regions. However, the same region must be always analyzed under the same settings in all samples. Among the parameters that must be standardized are the objectives and filters to be used, laser output, scan speed, pixel depth, scan direction, scan averaging, pinhole, zoom factor, amplitude offset and detector gain settings. Images must be optimized for contrast, brightness and background levels. Z-axis distance from the surface of the section must be standardized. Software that controls the LSCM is designed to facilitate these standardization procedures (i.e., parameters can be stored in the computer that controls the LSCM). Consult the manufacturer's instructions of each particular LSCM for details.
3. Fields should be selected randomly but within a standardized region. For instance, in a recent study on estrogen regulation of hippocampal NMDA receptor subunit expression, fields analyzed in the CA1 region were always located in the middle of this region (Gazzaley et al., 1996a). Special attention should be given to the prevention of fluorescence bleaching. Fields of interest should be located rapidly using either bright field or standard fluorescence illumination, making sure to set the lamp intensity to the lowest possible level that permits localization of the field. Make every attempt to expose all specimens to bright field or standard fluorescence illumination for the same length of time.

5.5.1.3 Potential Results, Analysis and Interpretation

Fluorescence intensity analysis of images obtained with an LSCM can be performed with software packages designed for each specific confocal microscope. Alternatively, images can be saved in a number of file formats that can be imported into many analysis programs such as Image-Pro® Plus (Media Cybernetics, L.P., Silver Spring, MD) or Bioquant® (R & M Biometrics, Nashville, TN). With the appropriate experimental and statistical analysis design, this type of experiment can detect relatively small differences in receptor levels. For instance, in the study of Gazzaley et al. (1996a), the authors obtained mean intensity values from six fields per region in three nonconsecutive hippocampal sections per animal and determined the percent change with respect to control. Data was subsequently analyzed by two-way ANOVA at a <0.05 level of significance and a Sheffe's *post hoc* test. Using this experimental design, it was possible to detect estrogen-induced increases of 10–20% in NMDAR1 subunit immunolabeling in the somatic and dendritic fields of the CA1 and dentate gyrus. Importantly, it was found in this study that estrogen did not affect expression levels of a housekeeping protein (i.e., MAP2), indicating that the effect of this hormone was specific for NMDAR1 subunits (Gazzaley et al., 1996a).

5.5.2 RADIOIMMUNOHISTOCHEMISTRY

5.5.2.1 Background

Radioimmunohistochemistry, also known as immunoautoradiography, is another method that can be used to perform quantitative analysis of receptor expression in tissue sections from ethanol-exposed animals. Studies have shown that this technique can provide accurate quantitative information on brain receptor protein expression levels. For example, Correa et al. (1991) demonstrated that quantification of angiotensin-converting enzyme in rat brain by enzymatic, ligand binding and radioimmunohistochemistry yielded comparable results. Using this technique, Eastwood and Harrison (1995) demonstrated a decrease in synaptophysin expression levels in samples from the medial temporal lobe of schizophrenics. More recently, Duelli et al. (1998) detected a decrease in the density of the Glut1 and Glut3 glucose transporters in visual structures of the rat brain during chronic deprivation, and Herman and Spencer (1998) showed adrenalectomy-induced effects on glucocorticoid receptor immunoreactivity in the rat hippocampus. This technique is also being used to assess the effects of prenatal ethanol exposure on metabotropic and ionotropic glutamate receptor expression in the hippocampus (Savage, unpublished observations). Figure 5.2 illustrates the distribution hippocampal AMPA receptors visualized by radioligand binding with ^3H -fluorowillardiine compared with the distribution of GluR subunits 1, 2/3 and 4 visualized by radioimmunohistochemical techniques. Whereas the distribution of antibody binding to subunits is similar in some regions, there are subtle differences in subunit distribution in other areas, as illustrated by examining GluR1 and GluR4 binding in the apical dendritic field of the hippocampal CA3 region. It is important to note that, while quantitative comparisons can be made in multiple brain regions and sections processed by the same radioimmunohistochemical procedure, quantitative comparisons of different antibody binding reactions are not possible using these methods.

5.5.2.2 Methodology

1. Tissue preparation, sectioning, fixing and incubation with primary antibodies can be performed as described in section 5.5.1.2.
2. Incubate sections with secondary immunoreagents. Radiolabeled species-specific secondary antibodies are frequently used for this type of procedure. Anti-mouse, -rabbit and -rat antibodies labeled with ^{125}I , or, more commonly, ^{35}S , can be purchased, for example, from Amersham (Piscataway, NJ) or NEN (Boston, MA). The molarity of a particular batch of antibody should be calculated using the specific activity and concentration information provided by the manufacturer. For instance, if an antibody batch has a specific activity of 600 Ci/mmol and a concentration of 0.1 $\mu\text{Ci}/\mu\text{l}$, then the molarity of this batch would be 166 nM. Make sure to correct for isotope decay as necessary. Optimal dilutions and incubation times for radiolabeled secondary antibodies should be determined empirically in a set of preliminary experiments. Start by testing 0.1–1 nM concentrations for 1 h at room temperature

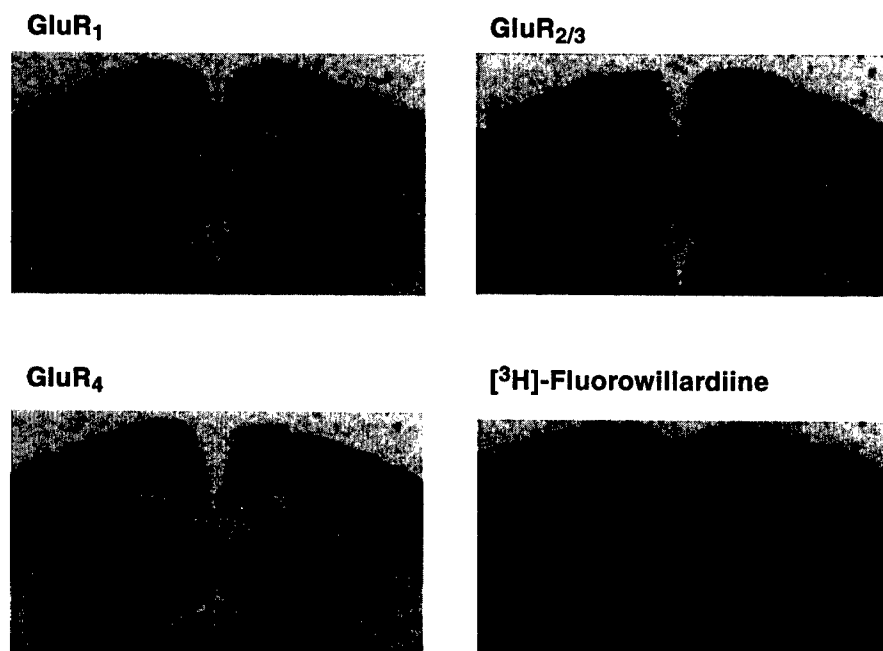


FIGURE 5.2 Radioimmunohistochemistry detection of immunolabeling with anti-AMPA receptor subunit antibodies, performed with coronal frozen sections from adult rats. Immunoaffinity purified polyclonal anti-GluR1, GluR2/3 and GluR4 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Donkey anti-rabbit ^{35}S -labeled secondary antibody was from Amersham-Pharmacia Biotech (Piscataway, NJ). For comparison, a ligand binding autoradiogram with the AMPA receptor ligand, ^3H -fluorowillardiine (Tocris, Ballwin, MO), is also shown.

in PBS plus 2–4% BSA (with 0.2–0.3% Triton X-100 if it was used to permeabilize sections before). Importantly, radiolabeled secondary antibodies should be tested in the absence of primary antibody to determine their background signal contribution.

3. Remove secondary antibody solution and dispose of radioactive waste according to the guidelines of your institutional radiation safety department. Wash sections 3×10 minutes with PBS at room temperature in Coplin or other type of jar. Dip three times in water and then air-dry sections.
4. Load sections into film cassettes along with standards containing known amounts of radioactivity. Standards can be created by blotting varying amounts of labeled secondary antibody onto nitrocellulose. Load film (for example, Kodak Biomax MR or MS film), close cassettes and store at -20°C during exposure.
5. For each new radioimmunohistochemical procedure, the film exposure period will need to be determined empirically and will vary as a function of receptor density and the specific activity and incubation concentration of secondary antibody.

6. Follow the manufacturer's instruction for developing the film. Dry the film overnight. For ease of handling and protection of the film emulsion, autoradiograms can be cut into strips and mounted onto glass microscope slides using Permount® (Fisher Scientific, Pittsburgh, PA). Images from mounted film sections can be acquired with a light microscope equipped with a digital camera.
7. Alternatively, radiolabeled sections can be exposed to screens that can be further analyzed by a phosphorimager or similar device (Charon et al., 1998).

5.5.2.3 Potential Results, Analysis and Interpretation

Densitometric analysis of radioimmunohistochemistry autoradiograms can be performed with programs such as the Image-Pro® Plus (Media Cybernetics, L.P., Silver Spring, MD). First, a standard curve of optical density for each standard of known radioactivity per unit area is generated. Then, antibody binding per unit area is determined by measuring the optical density and converting the data to moles per unit area based on the standard curve. For many brain regions, optical density measurements of ^{35}S -labeled antibody binding on the right and left side of four sections can be averaged to obtain a single measure of total antibody binding over each area of interest. The right and left side of two sections incubated in the absence of primary antibody can be used to obtain a single measure of nonspecific antibody binding. Specific ^{35}S -labeled antibody binding can be defined as the difference between total and nonspecific binding.

5.6 CONCLUSIONS AND FUTURE DIRECTIONS

In this chapter, we have described three methods that can be used to quantify the effects of ethanol on the protein expression levels of neurotransmitter receptor subunits. Using one or more of these methods in conjunction with ligand binding and mRNA-based techniques should provide important information on the short- and long-term effects of ethanol on the expression of neurotransmitter receptor subunits. A recent Pubmed search of the literature revealed that quantitative immunoblotting and immunohistochemical assays have been applied to study the effects of ethanol only on a very small subset of neurotransmitter receptor subunits, particularly to those belonging to the GABA_A and ionotropic glutamate family of receptors. Therefore, the effect of ethanol on the expression of subunits belonging to other families of neurotransmitter receptors remains an open question. We hope that this chapter kindles the interest of many investigators to pursue studies on this important area of alcohol-related neuroscience research.

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In vitro tissue slice preparations have proven particularly useful in alcohol research. Previous investigators have employed these methods in the initial discovery and subsequent characterization of the effects of ethanol on excitatory and inhibitory synaptic transmission. Tissue slices have been used to investigate ethanol modulation of synaptic transmission in areas ranging from the spinal cord (Wang et al., 1999) and brainstem (Eggers et al., 2000) to the cerebral cortex (Soldo et al., 1994; Sessler et al., 1998). In addition, synaptic effects of ethanol have been characterized in slices from a wide variety of species ranging from mice (Whittington and Little, 1990) and rats (Carlen et al., 1982) to monkeys (Ariwodola et al., 2000).

A number of features of the brain slice preparation make it an ideal model system with which to investigate the synaptic effects of drugs like ethanol. First, unlike most cell culture models, many of the interneuronal connections that form between different cell types are preserved. While not all afferent and efferent projections are maintained in brain slices, the synapses that are preserved have developed in their native environment and are representative of the anatomical relationships that are observed *in vivo*. These features provide the opportunity to characterize the effects of ethanol on native synaptic receptors. The importance of this feature is underscored by reports highlighting differences between the effects of ethanol on native and recombinant receptors (e.g., Sapp and Yeh, 1998) and even between the effects of ethanol on native receptors characterized in slices and in culture (Weiner et al., 1999; Costa et al., 2000). Second, the stability and experimental access afforded by the brain slice preparation greatly facilitates the application of techniques like single cell recording that are extremely difficult to carry out in intact animals. These same features also allow for much greater experimental regulation of the time course of drug applications as well as precise control of drug concentrations. Finally, since brain slices can be prepared from animals following chronic exposure and withdrawal of alcohol, these preparations provide the opportunity to assess *in vitro*, the neuroadaptive changes associated with alcohol abuse that develop *in vivo*.

Although brain slices can be prepared from virtually any area of the brain, the hippocampal slice has been, and continues to be, the most popular *in vitro* slice preparation. Therefore, many of the slice methods discussed in this chapter were originally developed for use in hippocampal tissue. Nevertheless, these methods can generally be adapted for use in preparing slices from other brain regions. The popularity of the hippocampal slice rests largely on its lamellar and laminar neuronal organization. Neurons in many brain regions are either sparsely distributed or localized in clusters containing many different cell types. In addition, most brain regions receive afferent inputs from a complex and dizzying array of sources, making it difficult to preserve and subsequently identify synaptic inputs in slices of these regions. In contrast, the hippocampus contains a number of large, homogenous populations of densely packed cells (Schwartzkroin, 1975; Dingledine et al., 1980). These cells form a well-described trisynaptic circuit that is essentially preserved in transverse sections made along its longitudinal axis (Andersen et al., 2000). In addition, much of the inhibitory control of this brain region arises from a diverse array of local circuit interneurons and this inhibitory circuitry is also well preserved in acutely prepared slices (Freund and Buzsaki, 1996).

8.2.1 PREPARATION OF BRAIN SLICES

Regardless of the brain region to be studied, the first steps in preparing brain tissue for electrophysiological investigation are similar. The animal to be used is sacrificed by decapitation and the brain is rapidly removed (no longer than 2 min) into a well-oxygenated ice-cold artificial cerebrospinal fluid (aCSF) solution. Animals of any age can be used. However, we and others have generally found that brain slices prepared from younger rats (2–6 weeks old) tend to be more viable and easier to record from (Lipton et al., 1995). We have found that, if slices are prepared from aged animals or from species in which the removal of brain tissue may take longer than a couple of minutes, transcardial perfusion with ice-cold aCSF prior to removal of the brain enhances slice viability.

Most studies use an aCSF that is some modification of a “Krebs” or “Ringer” type saline solution. The specific ingredients are often customized to the particular needs of the experiment (e.g., omitting Mg^{++} to record N-methyl-D-aspartate (NMDA) receptor-mediated responses). We have used the same basic aCSF recipe for many years with generally good results (Weiner et al., 1994; 1997a,b; 1999). This solution contains (in mM): 126 NaCl, 3 KCl, 1.5 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4 , 11 glucose, and 26 $NaHCO_3$. The aCSF is then saturated with 95% O_2 -5% CO_2 using a gas dispersion stone. This mixture ensures adequate oxygenation of the tissue and maintains the pH within a physiological range (7.3–7.5).

One variable that has been reported to alter some of the acute effects of alcohol in *in vitro* studies is the use of an anesthetic agent prior to sacrifice. For example, methoxyflurane and CO_2 anesthesia have been shown to disrupt the allosteric modulation of γ -amino-butyric acid_A (GABA_A) receptors by flunitrazepam and ethanol (Engel et al., 1996). Although rapid decapitation without anesthesia is permissible under the guidelines of the American Veterinary Medical Association (2001), the use of an anesthetic agent is recommended when it will not interfere with experimental outcomes. To date, the acute effects of ethanol on excitatory (Weiner et al., 1999) and inhibitory (Weiner et al., 1994; 1997a, b; 1999) synaptic transmission in brain slices that we have characterized do not appear to be influenced by the use of halothane anesthesia prior to decapitation. However, whenever characterizing novel effects of ethanol, it is often necessary to carry out an empirical evaluation to ensure that the use of an anesthetic does not interfere with the ethanol effects being investigated.

Once the brain has been removed and incubated in chilled, aerated aCSF for no more than 1 to 2 min, the tissue is then prepared for slicing. Two methods are commonly employed in the preparation of brain slices for electrophysiological recording. The first method involves using a manual (Figure 8.1A) or automated tissue chopper to section the tissue. In this method, the brain is hemisected and the region of interest is dissected free of surrounding tissue. The dissected tissue block is then secured on a piece of filter paper and positioned perpendicular to the blade of the chopper. The blade is usually a thin, double-edged carbon or steel razor adjusted such that it cuts into, but not through, the filter paper securing the tissue. These choppers are typically some variation of the first device developed for tissue slice preparation

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A number of methodological advances have played a central role in facilitating the transition in our current understanding of the mechanisms underlying alcohol's effects on CNS function. One key has been the development of electrophysiological techniques that enable the study of synaptic transmission in acute brain slice preparations. These methods were instrumental in first identifying the synapse as a central target of alcohol action. Moreover, these methods continue to be used by many investigators to unravel the complex mechanisms through which alcohol disrupts interneuronal communication.

The purpose of this chapter is to describe the methods involved in preparing brain slices for electrophysiological recording as well as some of the more common recording techniques in use today. The focus will be on providing practical insight into the application of these methods. Where possible, examples of how these techniques have been used in alcohol research will also be provided.

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8.2 THE *IN VITRO* TISSUE SLICE PREPARATION

Thanks to the pioneering work of Henry McIlwain and his colleagues, the acute brain slice preparation has become a mainstay in electrophysiological research. Ironically, this preparation was originally developed for use in biochemical studies characterizing energy metabolism in neuronal tissue. These early studies demonstrated that, under appropriate experimental conditions, acutely prepared slices of brain tissue could maintain normal levels of ATP and phosphocreatine for several hours (McIlwain et al., 1951). Soon after, McIlwain and his colleagues discovered that, in addition to demonstrating biochemical markers of viability, neurons in brain slices could actually maintain viable membrane potentials (Li and McIlwain, 1957). In addition, synaptic responses could be recorded in these preparations in response to electrical stimulation (Yamamoto and McIlwain, 1966). These early groundbreaking studies laid the foundation for countless numbers of investigators who have adapted and extended these *in vitro* methods to make seminal contributions to our understanding of the complex processes that underlie synaptic transmission.

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8 Electrophysiological Assessment of Synaptic Transmission in Brain Slices

Jeff L. Weiner

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8.1 INTRODUCTION

Over the last century, many advances have been made in elucidating the physiological mechanisms underlying the behavioral and cognitive effects of alcohol (ethanol). Early theories suggested that ethanol partitioned into neuronal membranes in a rather nonspecific manner, thereby causing a generalized disruption of central nervous system (CNS) function (Seeman, 1974; Goldstein, 1986). Over the past 30 years, it has become increasingly clear that ethanol perturbs neuronal function in a far more

selective manner than originally thought. It is now widely appreciated that acute exposure to alcohol directly alters the activity of some of the ligand-gated ion channels that mediate excitatory and inhibitory synaptic transmission (Faingold et al., 1998; Tsai and Coyle, 1998). Alcohol also disrupts the function of a select group of voltage-gated ion channels that regulate neurotransmitter release and neuronal firing (Brodie and Appel, 1998; Dopico et al, 1999; Walter and Messing, 1999). Moreover, these same synaptic proteins appear to undergo extensive neuroadaptive changes in response to chronic alcohol exposure and withdrawal.

A number of methodological advances have played a central role in facilitating the transition in our current understanding of the mechanisms underlying alcohol's effects on CNS function. One key has been the development of electrophysiological techniques that enable the study of synaptic transmission in acute brain slice preparations. These methods were instrumental in first identifying the synapse as a central target of alcohol action. Moreover, these methods continue to be used by many investigators to unravel the complex mechanisms through which alcohol disrupts interneuronal communication.

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A number of features of the brain slice preparation make it an ideal model system with which to investigate the synaptic effects of drugs like ethanol. First, unlike most cell culture models, many of the interneuronal connections that form between different cell types are preserved. While not all afferent and efferent projections are maintained in brain slices, the synapses that are preserved have developed in their native environment and are representative of the anatomical relationships that are observed *in vivo*. These features provide the opportunity to characterize the effects of ethanol on native synaptic receptors. The importance of this feature is underscored by reports highlighting differences between the effects of ethanol on native and recombinant receptors (e.g., Sapp and Yeh, 1998) and even between the effects of ethanol on native receptors characterized in slices and in culture (Weiner et al., 1999; Costa et al., 2000). Second, the stability and experimental access afforded by the brain slice preparation greatly facilitates the application of techniques like single cell recording that are extremely difficult to carry out in intact animals. These same features also allow for much greater experimental regulation of the time course of drug applications as well as precise control of drug concentrations. Finally, since brain slices can be prepared from animals following chronic exposure and withdrawal of alcohol, these preparations provide the opportunity to assess *in vitro*, the neuroadaptive changes associated with alcohol abuse that develop *in vivo*.

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Once the brain has been removed and incubated in chilled, aerated aCSF for no more than 1 to 2 min, the tissue is then prepared for slicing. Two methods are commonly employed in the preparation of brain slices for electrophysiological recording. The first method involves using a manual (Figure 8.1A) or automated tissue chopper to section the tissue. In this method, the brain is hemisected and the region of interest is dissected free of surrounding tissue. The dissected tissue block is then secured on a piece of filter paper and positioned perpendicular to the blade of the chopper. The blade is usually a thin, double-edged carbon or steel razor adjusted such that it cuts into, but not through, the filter paper securing the tissue. These choppers are typically some variation of the first device developed for tissue slice preparation

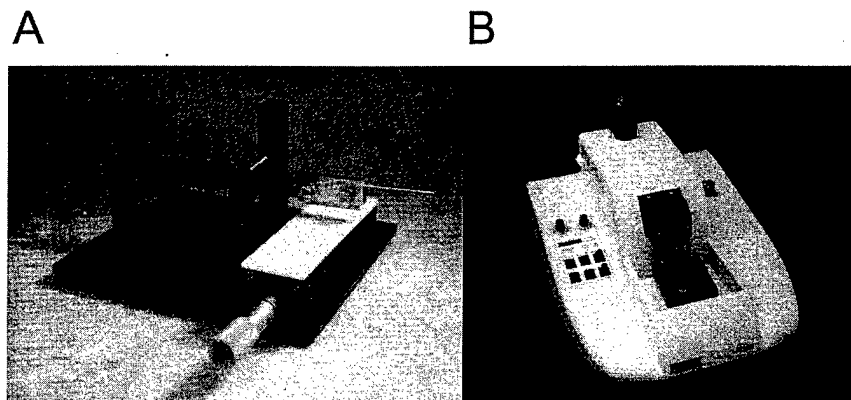


FIGURE 8.1 Photographs of instruments commonly used in the preparation of tissue slices for electrophysiological recording. (A) A manual tissue chopper (Stoelting, Wood Dale, IL) with an analog micrometer that is frequently used for preparing hippocampal slices for extracellular or “Blind” patch-clamp recordings. (B) A motorized, oscillating tissue microtome with digital section thickness controller (Leica Microsystems) commonly employed when preparing relatively thin (100–300 μm) sections for visualized cell recording. This model is designed to allow ice to be packed around the inner cutting chamber to keep the aCSF at a cold temperature during tissue sectioning.

by Henry McIlwain. The advantages of this method are that it is inexpensive, relatively fast (total slicing time < 5 minutes) and generally yields excellent slices. The disadvantages of these McIlwain-type choppers are that they are not practical for cutting uniform, thin tissue sections (< 300 μm), which are often necessary for visualized cell recordings (described in Section 3.3). Chopper methods may also not be suitable for slicing brain regions that are difficult to dissect free of surrounding tissue, such as the thalamus or striatum. Finally, we have found that this method, which requires considerable handling of the tissue, requires more training than using a vibrating tissue slicer (see below). However, for a well-trained investigator on a budget, tissue choppers produce excellent quality slices, particularly from hippocampus, that are suitable for most *in vitro* electrophysiological experiments.

The second option for slicing tissue is to use a motorized, vibrating tissue slicer (Figure 8.1B). In this method, the brain is rapidly removed as described above and a block containing the region of interest is fixed to a cutting stage with cyanoacrylate glue. After the tissue is secured, the stage is fastened to the base of a chamber filled with chilled, oxygenated aCSF and the tissue is sectioned using the vibrating, oscillating blade of the slicer. There are a number of makes and models of tissue slicers to choose from. All models provide some control of the cutting angle, speed, and oscillation frequency of the blade. In our experience, a blade angle of 10–15 degrees, a slow cutting speed and high oscillation frequency produce the best results. Of course, a slower cutting speed will prolong the slicing process. Therefore, a balance must be reached such that the blade advances slowly enough that it does not push the tissue and the slicing process takes no longer than 10–15 min. Another helpful tip is to keep the aCSF bathing the tissue as cool

as possible (by surrounding the cutting chamber with ice) and well aerated throughout this procedure.

There are several advantages of a vibrating tissue slicer. Most investigators report that these instruments provide better-quality tissue sections than the classical tissue choppers (Lipton et al., 1995). Vibrating slicers appear to cause less damage to the surface of the tissue, a factor that is particularly important when preparing thin sections for visualized cell recording (see section 3.3). As noted above, vibrating slicers are also better suited for preparing tissue sections from brain regions that are not readily dissected free from the whole brain. The disadvantages of tissue slicers are that they can be considerably more expensive than choppers and the slicing procedure usually takes longer than the chopper method.

8.2.2 SLICE INCUBATION AND RECORDING CHAMBERS

Once the tissue has been sectioned using either a chopper or vibrating slicer, it is immediately transferred to an incubation chamber. Most studies suggest that brain slices be allowed to equilibrate for a minimum of 1 h before recording (Lipton et al., 1995). The two main options for storing and recording from brain slices are interface and submersion chambers. In an interface chamber, the slices rest on a nylon mesh and are slowly perfused with oxygenated aCSF with the fluid level adjusted such that it just covers the surface of the tissue. The chamber is usually maintained between 32–35° C and warmed, moisturized O₂/CO₂ mixture is blown over the tissue. Some investigators have suggested that interface chambers provide the optimal method for storing and recording from acutely prepared brain slices (Lipton et al., 1995). Moreover, since the aCSF level of the bath is set to be just above the surface of the tissue, the tissue surface can be detected electrically once the recording electrode makes contact with the bath solution. This is particularly useful when using sharp intracellular electrodes as their tips can be very difficult to visualize. The only real disadvantage of interface chambers relates to their use as recording chambers in pharmacological studies, as the exchange of the bath solution can be extremely slow. Therefore, the use of interface chambers is not recommended in studies that require the frequent introduction and washout of drugs into the recording chamber.

The second option for storing and recording from brain slices is to use a submersion chamber. Submersion storage chambers are typically constructed from a simple beaker fitted with a mesh-covered grid (Figure 8.2). The beaker is filled with aCSF and continuously aerated with 95% O₂/5% CO₂. Slices are gently transferred to individual wells of the chamber using a polished Pasteur pipette and stored for the day's experiment. We have used submersion chambers such as the one illustrated in Figure 8.2 for many years and have found them to be very reliable for storing tissue sections. In a recent study on monkey brain slices (Ariwodola et al., 2000), viable recordings of dentate granule neurons, judged by an initial membrane potential more negative than –65 mV, were obtained from slices stored in a submersion chamber for up to 15 h. The primary advantage of submersion chambers relates mainly to their use as recording chambers. Submersion recording chambers usually have a relatively small volume (150–500 µL) and a faster aCSF perfusion rate (2 ml/min) than that of interface chambers. These

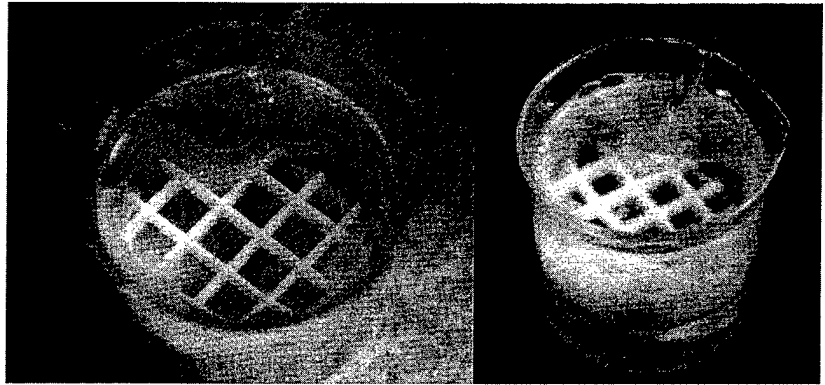


FIGURE 8.2 Submersion storage chamber for incubation of acutely prepared tissue slices. The chamber is constructed from a 500 mL beaker and is filled with aCSF that is continuously aerated with 95% O₂/5% CO₂ delivered via a gas diffusion stone. The chamber is fitted with a mesh-lined grid constructed from polyethylene filters (540μm thickness, 526μm openings) that serves to keep tissue sections separated during storage. The temperature of the chamber can be adjusted using a heated water-bath.

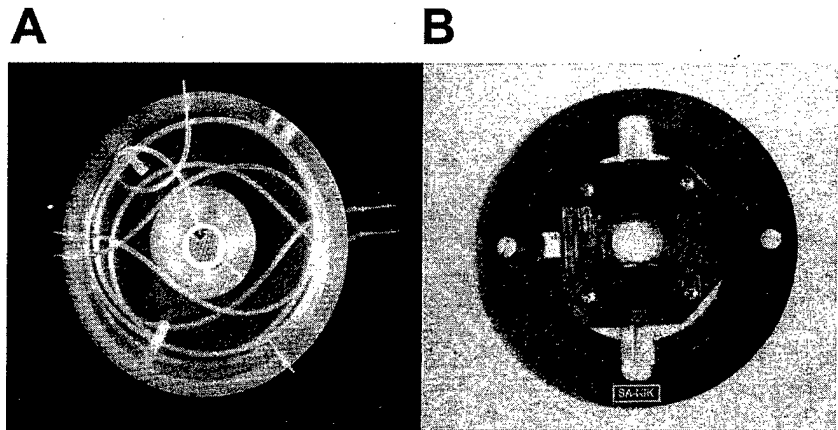


FIGURE 8.3 Common submersion recording chambers used in tissue slice electrophysiological studies. (A) A custom-made Haas-type chamber in which the tissue rests on a nylon-mesh that is fitted into a raised chamber in the center of the bath. The tissue is perfused from above and below with aerated aCSF. The outer chamber is equipped with a nichrome wire that can be used to heat the aCSF perfusing the recording chamber. (B) Glass-bottom chamber used in visualized slice recording (Warner Instruments, Hamden, CT). The tissue is weighted down using small platinum wire weights and the chamber can be heated by warming the perfusate through an external water bath prior to perfusion of slices.

factors greatly facilitate the application and washout of drugs. Therefore, most electrophysiological studies that focus on characterizing the effects of drugs, like ethanol, on synaptic transmission generally employ a submersion chamber, at least for recording. Two common styles of submersion chambers are illustrated in Figure 8.3.

One important variable regarding the storage and recording of brain slices involves the temperature at which slices are maintained. It may seem intuitive that storing and recording from slices at, or near, physiological temperatures would be desirable for most experiments. However, for practical reasons, many electrophysiological studies are carried out at room temperature. In fact, even when trying to carry out experiments at physiological temperatures, most investigators opt to record at temperatures slightly below those observed *in vivo* (32–35° C). We have found that, regardless of the temperature at which experiments will be carried out, it is best to maintain the tissue slices at a constant temperature throughout storage and recording. In rat hippocampal slices, gradually increasing the temperature following the dissection from 13° C to ambient levels can result in profound changes in a variety of electrophysiological parameters (Watson et al., 1997) and in basal protein kinase C activity (Weiner et al., 1997b).

8.2.3 STIMULATION AND PHARMACOLOGICAL ISOLATION OF SYNAPTIC RESPONSES

As mentioned earlier, one of the most powerful advantages of brain slices is that functional synapses are preserved in these preparations. Therefore, electrical stimulation of afferent inputs can generate excitatory and inhibitory synaptic responses. These responses can be recorded extracellularly from large groups of cells or intracellularly from individual neurons. Stimulation is usually delivered using relatively small metal electrodes. We employ concentric (Figure 8.4A) or bipolar (Figure 8.4B) tungsten wire electrodes that provide a relatively discrete stimulation focus. Bipolar electrodes can also be fashioned by pulling glass theta tubing to a relatively thin tip (1–2 μ m). Both sides of the electrode are then filled with a concentrated electrolyte solution (e.g., 3M NaCl) and connected to a stimulus isolation unit with thin wire connectors (Banks et al., 1998; Jensen and Mody, 2001). These glass electrodes are particularly useful for triggering monosynaptic responses from defined morphological zones (e.g., somatic vs. dendritic synapses).

Because most slices contain a variety of functional excitatory and inhibitory synaptic inputs, electrical stimulation typically generates complex synaptic responses mediated by multiple ion channels. In many studies, the desired goal is to characterize the effects of a drug on an individual element of synaptic transmission, such as NMDA receptor-mediated synaptic excitation or GABA_A receptor-mediated synaptic inhibition. This can usually be achieved by using selective receptor antagonists to “pharmacologically isolate” the synaptic response of interest. For example, GABA_A receptor-mediated inhibitory synaptic responses in most brain regions can be effectively isolated using a cocktail of GABA_B, NMDA, and AMPA/kainate receptor antagonists. Unfortunately, the identity of all synaptic elements may not always be known and appropriate pharmacological tools may not be readily available. For example, it has been known for many years that kainate

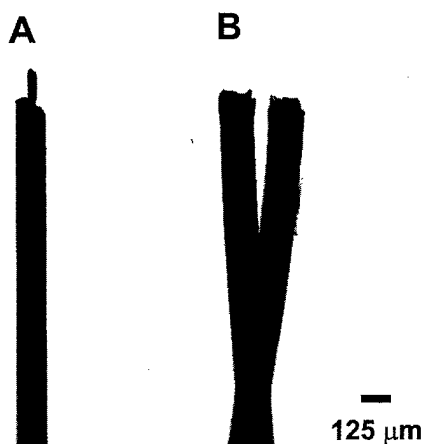


FIGURE 8.4 Stimulating electrodes used to evoke synaptic responses in brain slices. (A). Concentric bipolar stimulating electrode constructed from a 125 μm stainless steel outer pole and a 25 μm platinum/iridium inner pole (FHC, Bowdoinham, ME). (B) Twisted tungsten wire bipolar electrode (Plastics1, Roanoke, VA) with a slightly larger tip ($\sim 200 \mu\text{m}$).

receptors are highly expressed in the rat hippocampus (Foster et al., 1981). However, the lack of an antagonist that can distinguish between kainate receptors and their more prominent relatives, the AMPA receptors, precluded any assessment of the physiological role or pharmacological profile of these receptors in tissue slices. The relatively recent development of AMPA receptor-selective antagonists (Bleakman et al., 1995) sparked a flurry of studies demonstrating a number of novel pre- and postsynaptic roles for kainate receptors in the hippocampus and other brain regions (see Ben-Ari and Cossart, 2000 for review). We used one of these selective AMPA receptor antagonists, LY 303070, to pharmacologically isolate a kainate receptor-mediated synaptic current in hippocampal CA3 pyramidal neurons and characterized its sensitivity to ethanol (Figure 8.5). Surprisingly, this study revealed that, although ethanol had no effect on AMPA excitatory postsynaptic currents (EPSCs), as shown by many others (Faingold et al., 1998; Tsai and Coyle, 1998), it potently inhibited kainate receptor-mediated synaptic responses in these cells (Weiner et al., 1999). We have since identified a number of other kainate receptor-mediated responses that are also inhibited by ethanol in the rat hippocampus (Crowder et al., 2000) and nucleus accumbens (Crowder and Weiner, 2001).

Because the majority of slice electrophysiology experiments require the introduction of at least some drugs into the bath, a number of methods have been developed to accomplish this process. The most common approach is simply to exchange the solution bathing the tissue with one containing the desired drug(s). A number of reservoirs can be set up with different combinations of drugs or varying concentrations of a single drug, like ethanol. Each reservoir must be equipped with a gas diffusion stone to provide continuous aeration with the O_2/CO_2 mixture. Solution flow is usually driven by gravity and switching between reservoirs can be accomplished by manual or electrically controlled valves. When several reservoirs are used, their fluid levels and height should all be similar to

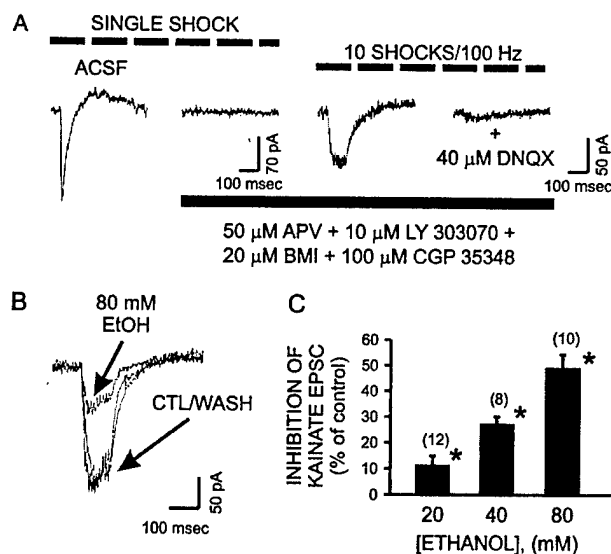


FIGURE 8.5 (A) Pharmacological isolation of slow kainate EPSCs in rat hippocampal CA3 pyramidal neurons. Note that in normal aCSF, individual stimuli evoke a biphasic response that is completely blocked a cocktail containing an NMDA receptor antagonist (APV), an AMPA receptor antagonist (LY303070), a GABA_A antagonist, and a GABA_B antagonist. In the presence of this cocktail, a stimulus train of 10 pulses delivered at 100Hz evokes a slow inward synaptic current that is then blocked by a mixed AMPA/KA receptor antagonist (DNQX). (B) Averages of four to seven pharmacologically isolated KA EPSCs evoked prior to, during, and after a 6-min application of 80 mM ethanol. (C) Bar graph summarizing the concentration dependence of ethanol inhibition of KA EPSCs. Numbers in brackets indicate the number of cells tested under each condition; *, $p < 0.05$. (Modified with permission from Weiner et al., 1999.)

ensure a constant flow rate into the bath. Roller bar perfusion pumps can be used instead of gravity to control the flow rate of the different solutions. Care must be taken to eliminate any pulsation introduced by these pumps as the mechanical interference can disrupt the recording.

We employ an alternative method that involves introducing drugs into the recording chamber via calibrated syringe pumps. In this method, adapted from Tom Dunwiddie's laboratory, an enclosed perfusion system is set up to provide a continuous, regulated flow of aCSF into the recording chamber. The perfusion is driven by pressure created by the O₂/CO₂ gas mixture aerating the aCSF. The flow rate is adjusted to 2 ml/min and this rate is continuously monitored using a calibrated flowmeter (Barnant Company, Barrington, IL). Concentrates of drugs (50–200X) to be introduced into the bath are mixed with the aCSF in a small port near the recording chamber via calibrated syringe pumps. The final bath concentration of each drug is controlled by the flow rate of each syringe pump. The main advantage of this perfusion method is that multiple concentrations of a drug can be introduced into the recording chamber from a single syringe, simply by altering the speed of the

syringe pump. Also, by placing the mixing port close to the recording chamber, drug exchanges can be achieved relatively quickly.

8.3 RECORDING TECHNIQUES

A number of electrophysiological methods have been developed for the study of synaptic transmission in brain slices. The first techniques enabled the detection of extracellular electrical activity from large numbers of neurons. Subsequent advances permitted recording synaptic potentials from individual cells. All of these techniques are still in use today and have a broad number of applications in alcohol research.

8.3.1 EXTRACELLULAR RECORDING

This technique involves recording the electrical activity of large populations of neurons by placing recording electrodes in the extracellular space adjacent to the target cells of interest. Extracellular recording is usually carried out with low resistance glass microelectrodes filled with concentrated NaCl or NaAcetate (3–10 mM; 5–10 M Ω tip resistance). The shape of these electrodes is similar to that of patch-clamp electrodes (Figure 8.6A), although extracellular electrode tip diameters may be slightly smaller to reduce leakage of the pipette contents. Extracellular signals are generally very small as they reflect the flow of ionic current through the relatively low resistance of the extracellular fluid. Because these responses are typically on the order of 10–500 μ V, they require fairly extensive amplification to be detected, making low instrument noise a critical technical consideration in the successful application of this technique.

Extracellular recording offers several advantages over intracellular recording methods. First, this technique is relatively simple and inexpensive to carry out. Because electrodes are placed in the extracellular space and not onto individual neurons, relatively inexpensive manipulators and stereomicroscopes provide adequate stability and visualization for these kinds of experiments. Moreover, although extracellular signals require extensive amplification, amplifiers that can perform these functions are generally much less expensive than those required for single cell recording. Other important advantages of this technique are that it can be carried out for relatively long periods of time and extracellular responses tend to be more stable and less variable than those recorded from individual cells, as they represent the summed activity of large populations of neurons. The stability and relatively low variability of these recordings makes them particularly well suited for studies that require long-term monitoring of a synaptic responses, such as during alcohol withdrawal (Thomas et al., 1998B) or when carrying out inter-subject comparisons (Nelson et al., 1999). Finally, some extracellular recording methods have been adapted to record extracellular activity *in vivo* in anesthetized and awake, freely moving animals (see Chapters 9 and 10).

However, several limitations of extracellular recording methods exist. Typically extracellular signals can be detected only in brain regions in which the activity of large populations of neurons is highly synchronized and the dipole orientation of the cells is uniform. Therefore, compound action potentials (population spikes) or field excitatory postsynaptic potentials (fEPSPs) from cortical or hippocampal

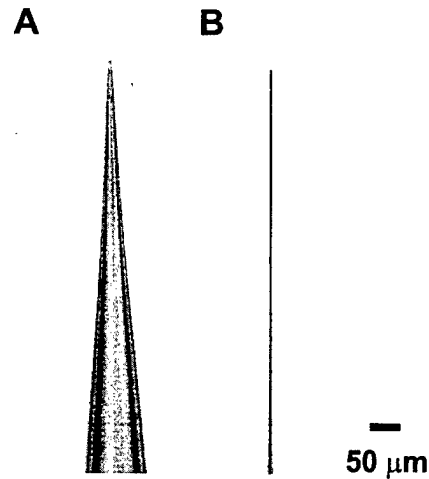


FIGURE 8.6 Glass micropipettes used for single-cell recording. (A) Patch microelectrode designed to record whole-cell responses using “blind” or visualized cell patch-clamp recording. (B) Microelectrode fashioned for intracellular current clamp recording. Note that the intracellular electrode tip diameter is only slightly smaller than that of the patch electrode, however the extended narrow taper results in a much higher tip resistance (Patch electrode = 8 MΩ; Sharp electrode = 135 MΩ, both measured with a 150 mM Kgluconate filling solution). Both electrodes were pulled using a horizontal, Flaming-Brown type puller using filamented, borosilicate glass (outer diameter = 1.5 mm, inner diameter = 0.86 mm) (both from Sutter, Novato, CA).

pyramidal cells are readily detected using extracellular recording methods. However, recording GABAergic inhibitory synaptic potentials through extracellular methods is considerably more difficult due to the diffuse density of these currents and their short dipoles. Extracellular methods can be used to assess GABAergic inhibition indirectly in some brain regions like the hippocampus where GABAergic synapses mediate a potent feed-forward inhibition of excitatory synaptic responses. This inhibition can be readily detected by measuring the inhibition of the second of two excitatory responses evoked at short interstimulus intervals (known as paired-pulse inhibition).

Extracellular recordings were used in one of the first demonstrations that intoxicating concentrations of ethanol did, in fact, alter synaptic transmission in the mammalian CNS. Durand and co-workers (1981) demonstrated that ethanol significantly attenuated orthodromically evoked population spikes in the rat hippocampal CA1 region. Using extracellular techniques, they showed that this inhibition was not due to a direct effect of ethanol on the voltage-gated channels that underlie action potentials, since antidromically evoked spikes, triggered by directly stimulating the axons of the CA1 pyramidal cells, were unaffected by ethanol. Extracellular recording methods were later used in the first demonstration that ethanol specifically inhibited the NMDA receptor-mediated component of excitatory synaptic transmission in the rat hippocampus (Lovinger et al., 1990). These techniques have also

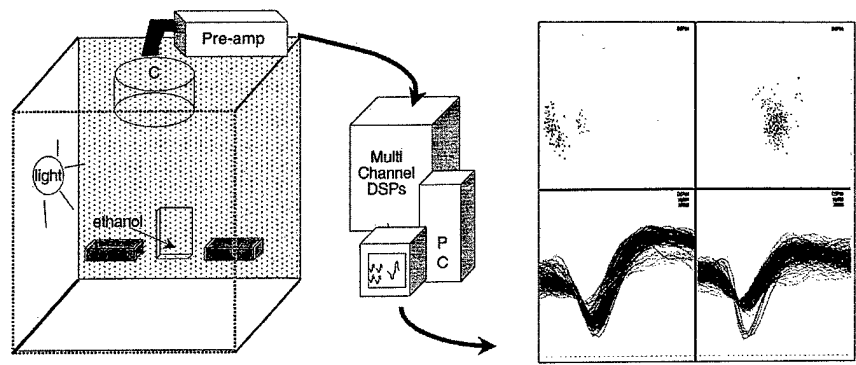


FIGURE 10.1

In vivo multichannel recording setup. Neural signals are acquired from rats during alcohol self-administration within a customized operant behavior chamber. Commutator (C) installed in center of chamber ceiling turns as the animal turns, and is designed to preserve integrity of signals from each channel while allowing free movement of subject throughout experimental chamber. C is connected to a preamplifier that amplifies and filters signals. Neural signals are then sent through analog-to-digital boards, where additional amplification or filtering may take place, then to digital signal processing (DSP) boards under the control of a personal computer (PC). Signals from each channel are sorted into waveforms that correspond to action potentials from individual neurons using specialized data acquisition software running on the PC. Examples of waveforms visible on two individual channels (wires) are shown on right. Two distinct waveforms are visible on each wire. Directly above waveforms is principal component analysis for each wire, which aids in sorting and confirms separation of the waveforms into two separate units because two separate clusters are visible. Digitized and sorted waveforms and timestamps (time of occurrence of each waveform) are then saved to PC along with times of occurrence of any behavioral events.

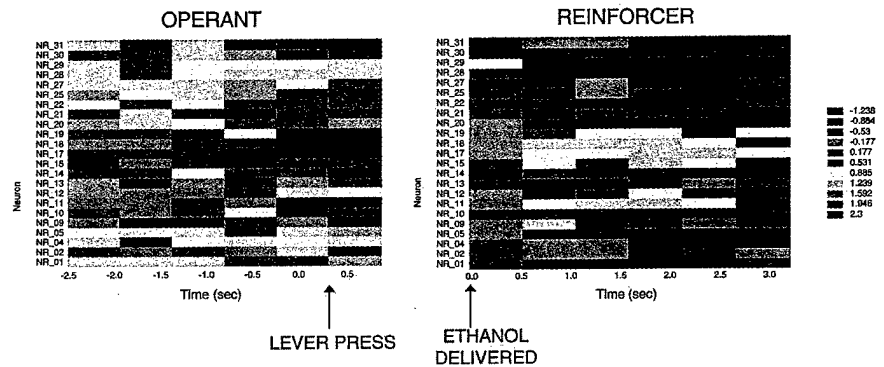


FIGURE 10.7

Patterns of neuronal activity within NAC are different during appetitive and consummatory phases of self-administration trial. Standardized firing rates of 21 simultaneously recorded NAC neurons are depicted relative to lever press response and alcohol receipt and consumption periods for a single alcohol self-administration session. Each neuron is represented in one row of contour plots, while time, in 500-msec blocks, is arranged across abscissa. There are more excitatory responses during appetitive phase of trial.

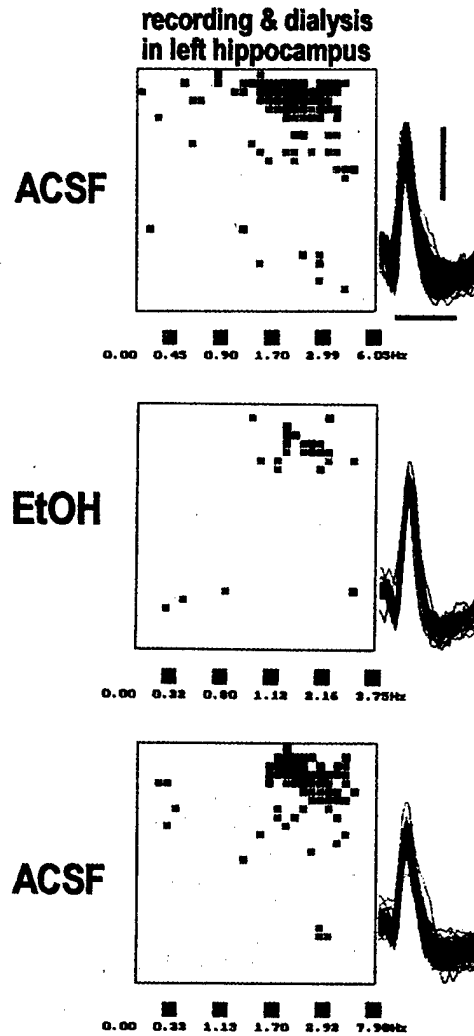


FIGURE 11.8

The effect of ethanol, reverse-dialyzed in the hippocampus, on the location-specific firing of a place cell in the microdialysis area. Firing rate distribution maps are shown, generated with the MapMaker software. The maps indicate the average firing rate of the place cell within the areas of pixels during 15-min data collection periods, as the rat moved around chasing food pellets on the floor of a rectangular test chamber. Black pixels represent areas where the cell fired with the highest firing rates; yellow pixels represent areas where the cell did not fire at all, and white areas represent areas not visited by the animal. During microdialysis with ACSF (upper panel), the cell displayed a clear location-specific firing (6.05 Hz) in the northern part of the chamber. When ethanol (1 M for 30 min) was perfused (middle panel), the location-specific firing rate of the cell dropped to 3.75 Hz. After washing out ethanol from the recording site, full recovery of the original location-specific firing (7.90 Hz) was observed (lower panel). The similarity of the corresponding overlaid action potential waveforms on the right of each panel indicate that the data were collected from the same place cell throughout the recording and dialysis session. Calibrations: horizontal bar: 0.5 msec; vertical bar: 0.1 mV.

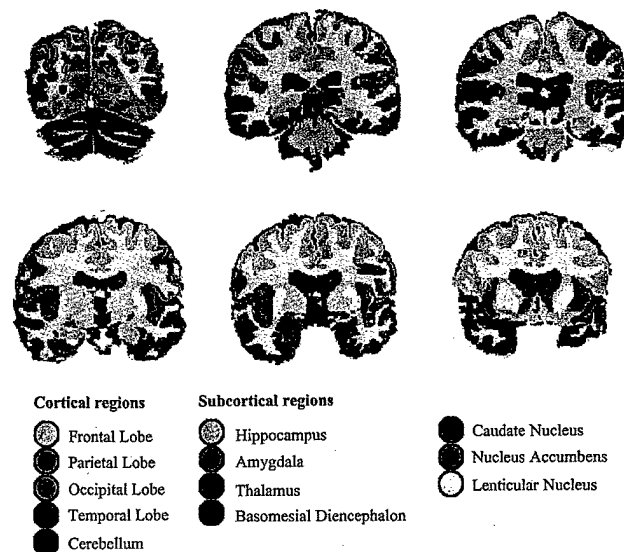


FIGURE 13.4

A set of "processed" MR images from a single individual studied in an MR morphometry study performed in the author's laboratory. The different regions and structures examined are color-coded to show the structural boundaries.

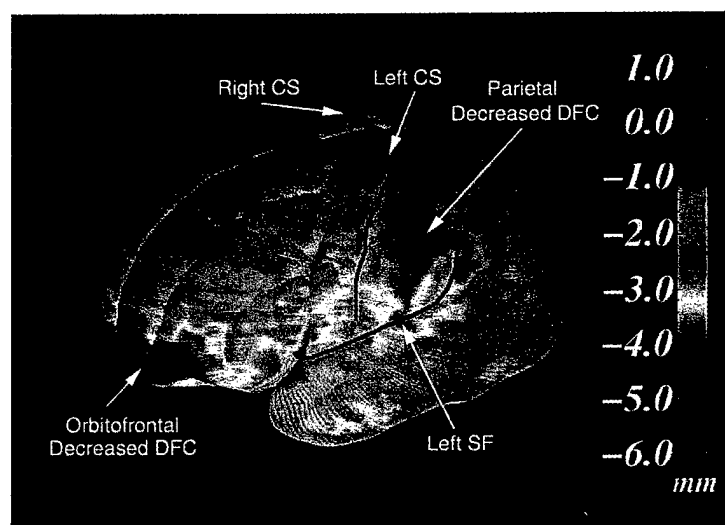


FIGURE 13.5

DFC group-difference maps showing differences in DFC (in mm) between the alcohol-exposed and control subjects according to the color bar on the right. Note negative effects in nearly all regions, most prominent in parietal and orbital frontal regions, i.e., DFC is greater in controls than in alcohol-exposed patients, with regional patterns of DFC reduction up to 8 mm. While only left hemisphere is fully visualized here, DFC reduction in both parietal and orbital frontal regions is bilateral. Left and right central sulcus (CS) and left hemisphere Sylvian fissure (SF) are highlighted in black.

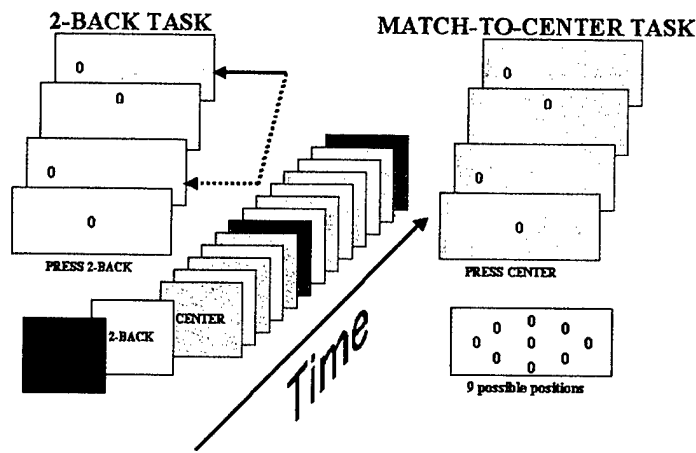


FIGURE 14.7

A pictorial representation of a block design, spatial working memory experiment and its three conditions: rest (green), 2-back task (yellow), and match-to-center attentional task (aqua). In the 2-back task, subjects were instructed to press a response key when a 0 appeared in the same position as one presented two presentations back. In the match-to-center task, subjects pressed a response key when a 0 appeared in the middle.

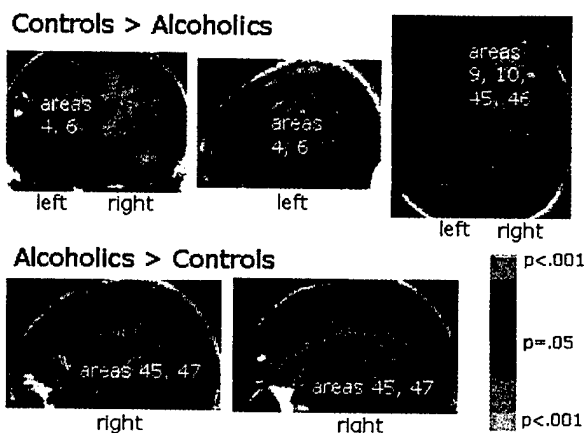


FIGURE 14.8

An example of SPM-derived significant activation clusters of differences between the attention task and a rest condition. Loci where the activation of the control group was significantly greater than that of the alcoholic group are depicted in the red to yellow tones. Loci where the activation of the alcoholic group was significantly greater than that of the control group are depicted in the blue to purple tones. Activation of control group was greater than that of alcoholic group in anterior medial and superior prefrontal cortex, where a large region of activation included Brodmann areas 9, 10, 45, and 46 bilaterally, although more widespread in the right than left hemisphere; the left motor cortex (areas 4 and 6) was also significantly more activated. By contrast, activation of the alcoholic group was greater than that of the control group in the right prefrontal cortex (areas 45 and 47), positioned more inferiorly and posteriorly than prefrontal regions significant in the controls. The differences in activations between the groups were due to greater activation in one group rather than deactivation in the other group (Figure 4 in Pfefferbaum et al., *Neuroimage* 14: 7-20, 2001).

proven invaluable in studies investigating the effects of fetal alcohol exposure (Bellinger et al., 1999) and chronic alcohol exposure and withdrawal on excitatory (Morrisett, 1994; Nelson et al., 1999) and inhibitory (Rogers and Hunter, 1992; Kang et al., 1996) synaptic transmission.

8.3.2 INTRACELLULAR (SHARP) ELECTRODE RECORDING

With the landmark demonstration that synaptic responses could be evoked in acutely prepared brain slices and recorded with extracellular techniques (Yamamoto and McIlwain, 1966), many investigators adopted these preparations to characterize the physiology and pharmacology of synaptic transmission. The subsequent demonstration that intracellular recording techniques, initially developed to study invertebrate neurophysiology and mammalian peripheral neurons, could be applied in brain slices to study the excitability and synaptic regulation of individual neurons, solidified the role of the brain slice as an invaluable tool in electrophysiology.

While classical "sharp electrode" intracellular recording is less frequently used today since the development of the patch-clamp technique, a number of important applications for this technique still exist. In this method, a glass microelectrode is used to impale an individual neuron, thereby providing a direct measurement of the electrical potential across the cell membrane. Intracellular electrodes are usually fashioned from borosilicate glass using methods similar to those used to prepare extracellular electrodes. However, thinner walled glass is usually employed and the shape of intracellular electrodes must be adjusted so that they can penetrate neurons without causing excess mechanical damage. Intracellular electrodes must have a homogeneously tapered shank that ends in a tip that is usually less than 1 μm (Figure 8.6B). Electrodes are filled with a concentrated electrolyte solution (usually 3–10 M KCl or KAcetate) and have a tip resistance between 60–120 M Ω . The electrode is advanced through an area of tissue with a high density of cells of interest (e.g., hippocampal pyramidal layer, cortical layer, dentate gyrus granule cell layer). The electrode movement is controlled using a manipulator that allows for high velocity micro-steps with minimal lateral oscillations. This is best achieved with stepping motor driven devices or piezoelectric manipulators. This rapid acceleration has been found to be necessary for successful penetration of the cell membrane (Sonnhof et al., 1982). While the electrode is advanced through the tissue, a small current pulse is injected into the electrode to monitor the tip resistance. Cells are "detected" electrically as a small increase in the resistance of the electrode. Once a cell is detected, the electrode is then advanced further, puncturing the membrane of the cell. Successful membrane penetration is immediately evidenced by a rapid drop in the electrode voltage, as the cytosolic potential is significantly more negative than that of the extracellular space. As this technique requires that the electrode actually puncture the cell membrane, it is often necessary to wait several minutes following membrane impalement while the membrane seals up around the intracellular electrode. This can be monitored by measuring the input resistance of the cell. Once the input resistance has stabilized, stable intracellular recordings can often be maintained for several hours.

One important feature of the intracellular recording configuration is that the cytosolic contents of the cell being recorded are largely preserved. This contrasts with the whole cell patch-clamp recording method discussed below, in which the contents of the cytosol may be extensively dialyzed. As a result, the use of intracellular electrodes may be preferable when characterizing the activity of ion channels that are particularly sensitive to modulation by second messenger systems, as their activity could be compromised during whole cell patch-clamp recording.

The major disadvantage of this technique is that, for cells to survive impalement, intracellular electrodes must have a small tip diameter and an extended, tapered shank. This electrode profile results in a relatively high tip resistance, making it impractical to measure the actual currents underlying synaptic transmission (voltage-clamp configuration), particularly in highly arborized neurons in brain slices. However, when used as a "voltage follower," intracellular electrodes perform very well.

The fine, tapered shape of intracellular electrodes does offer one important advantage over patch-clamp electrodes. Under appropriate experimental conditions, these electrodes can be used to carry out intracellular recordings in anesthetized animals *in vivo* (O'Donnell and Grace, 1995; Reynolds and Wickens, 2000; Aksay et al., 2001; Goto and O'Donnell, 2001). Such experiments, while technically demanding, provide one of the most sensitive ways to characterize synaptic transmission under conditions where all afferent and efferent inputs are preserved. Although patch-clamp recordings have been achieved in intact animals (Light and Willcockson, 1999), the shape of these electrodes limits their utility *in vivo*.

The intracellular recording technique was used to carry out the first detailed analysis of the effects of ethanol on excitatory and inhibitory components of synaptic transmission in the mammalian CNS. Carlen and co-workers (1982) used this method to characterize the acute effects of ethanol on synaptic transmission in rat hippocampal CA1 pyramidal neurons. In their studies, 10-20 mM ethanol, applied by superfusion or local microdrops, significantly enhanced excitatory and inhibitory synaptic potentials in the majority of cells recorded. Importantly, ethanol had minimal effects on the membrane potential and firing properties of these cells. Five years later, Siggins and co-workers (1987) carried out a similar study and confirmed that the predominant effect of ethanol was on synaptic transmission rather than on the passive or active membrane properties of the CA1 pyramidal cells. Unfortunately, they observed that ethanol, more often than not, inhibited rather than potentiated EPSPs and inhibitory postsynaptic potentials (IPSPs). Although these two studies differed in some of their conclusions regarding the effects of ethanol on synaptic transmission, they did agree that ethanol produced its most significant effects at the level of the synapse, rather than by directly altering the passive and active membrane properties of the principal neurons.

Intracellular recording techniques continue to be used today to study the effects of ethanol on neuronal activity. For example, this method was recently used to demonstrate that, in locus coeruleus neurons recorded in pontine slices, ethanol significantly inhibits EPSPs while having no significant effects on IPSPs or membrane properties of these cells (Nieber et al., 1998). Evans and colleagues (2000) recently used intracellular recordings in inferior colliculus neurons to characterize hyperexcitability associated with ethanol withdrawal. Finally, although ethanol does

not seem to have pronounced effects on the resting membrane potential of most neurons, there are a few exceptions. Brodie and Appel (1998) have used intracellular recordings to demonstrate that ethanol can directly depolarize and increase the firing rate of dopamine cells in the ventral tegmental area, an effect that may play an integral role in the rewarding properties of this drug.

8.3.3 WHOLE-CELL PATCH-CLAMP RECORDING

The patch-clamp recording technique was originally developed by Sakmann and Neher to study the biophysical properties of individual ion channels in small patches of muscle fiber (Neher et al., 1978). Soon this method was used to obtain high-resolution whole-cell recordings from isolated or cultured cells (Hamill et al., 1981). The major advance for synaptic electrophysiologists came with the application of this method to the study of individual neurons in brain slices (Edwards et al., 1989). Since these initial studies, the use of the patch-clamp technique has revolutionized the way that ion channel activity is studied. Although many of the details of patch-clamp recording have already been discussed in Chapters 4 and 7, several methodological considerations related to the application of this recording technique in brain slices warrant some discussion.

Each of the two general methods of obtaining whole-cell patch-clamp recordings in brain slices has its own advantages and disadvantages. In the "Blind" method, a patch electrode is advanced through a brain region in which there is a relatively dense, homogenous population of cells. Positive pressure is applied to the patch electrode to prevent membrane debris from blocking the electrode tip as it is advanced through the tissue. The electrode resistance is monitored in current-clamp mode by passing a small current step across the tip of the electrode. Much like in intracellular recording, cells are detected as an increase in the resistance across the tip of the electrode. Once a cell is detected, the positive pressure is removed, often resulting in a rapid increase in tip resistance due to the sealing of the cell membrane onto the electrode tip. If this does not occur spontaneously, a small amount of negative pressure is gently applied to the tip to facilitate seal formation. Injecting a small amount of negative DC current through the patch electrode seems to facilitate seal formation. Once a seal of at least 1 G Ω (preferably 3-5 G Ω s) is formed, an additional application of negative pressure is applied, resulting in the sudden rupture of the membrane between the electrode tip and the cell.

There is considerable debate among "patch-clampers" about whether the various pressure applications are best administered by mouth or via a syringe. Through many years of trial and error, we can unquestionably state that, when recording in brain slices, a syringe is adequate for maintaining pressure while in the "cell search" mode. However, seal formation and "going whole-cell" are more consistently achieved using experimenter-controlled oral pressure application.

The primary advantage of the "Blind" patch method is that it is relatively inexpensive to set up, as a simple dissecting microscope provides adequate magnification (Figure 8.7a). In addition, a recording chamber that provides superfusion of both sides of the tissue slice can be used (Figure 8.4A). We have generally found that tissue slices seem to remain viable for longer periods of time in these kinds of

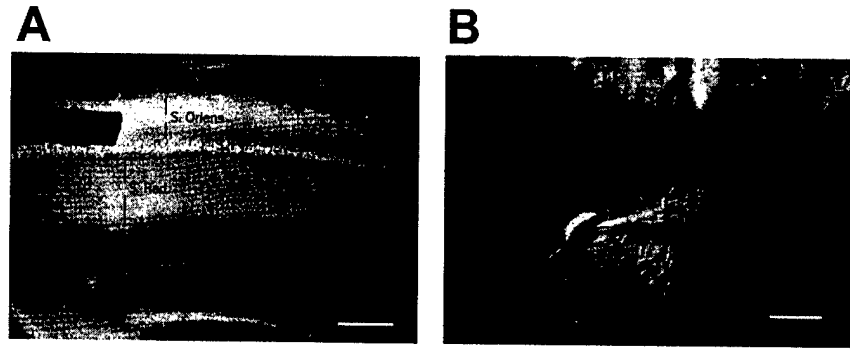


FIGURE 8.7 (A) Low-power view of hippocampal slice preparation showing location of a bipolar stimulation electrode (top left) as well as a patch-clamp recording electrode located in the stratum radiatum of the CA1 region. Scale bar, 100 μm . (B) A high-power DIC image of a single stratum radiatum interneuron, located $\sim 100 \mu\text{m}$ below the surface of the slice, during recording with a patch-clamp electrode. The pyramidal cell layer is visible at the top of the view. Scale bar, 10 μm . (Reproduced with permission from Frazier et al., 1998; Copyright 1998 by the Society for Neuroscience.)

slice chambers compared with the glass-bottomed submersion chambers used in visualized cell recording (Figure 8.4B). This is particularly noticeable when recording at more physiological temperatures ($32\text{--}35^\circ \text{C}$). The main disadvantage of the “Blind” patch method is that, as the name implies, visualization of the cell being recorded is not achieved. This is not a significant problem when recording from a densely packed, homogenous cell population like CA1 pyramidal neurons. This method would not, however, be very effective for recording from a sparsely distributed neuronal type such as hippocampal GABAergic interneurons.

The alternative method of whole-cell patch-clamp recording in brain slices involves directly visualizing the target neuron prior to recording. This can now be readily achieved by using a number of image enhancement techniques that compensate for the poor imaging qualities of brain slices. The first requirement is a microscope that is equipped with differential interference contrast (DIC) optics (Dodt and Zeiglgansberger, 1990). The DIC objective converts phase gradients created by the light-scattering properties of tissue slices into amplitude gradients and essentially allows for optical sectioning of relatively thick specimens ($100\text{--}300 \mu\text{m}$). Optical sectioning permits visualization of cells that may be located relatively deep within a tissue slice (Figure 8.7B). Additional improvement of image quality can be achieved using video enhancement technology (Allen and Allen, 1983) and by using infrared illumination. Infrared light penetrates through thick tissue sections better than visible light, serving to further aid in the visualization of individual cells (Dodt and Zeiglgansberger, 1990). In the DIC patch-clamp method, the recording electrode is positioned above the tissue and a cell of interest is visually identified. The recording electrode is then visually guided to the surface of the neuron using a mechanical or motorized micromanipulator. The remaining steps involved in forming a seal and gaining electrical access to the cell are identical to those described above for “Blind” patch-clamp recording.

The obvious advantage of this technique is that it enables the application of the whole-cell patch-clamp recording technique to be applied on virtually any type of neuron, no matter how sparsely distributed (e.g., hippocampal GABAergic interneurons; Figure 8.7B). Moreover, this technique has made it possible to stimulate (Banks et al., 1998) and actually record from (Stuart et al., 1997) dendritic processes in tissue slices. While DIC-video enhanced microscopy is unquestionably revolutionizing the use of brain slices in electrophysiology, there are several disadvantages of this method compared with "Blind" patch-clamp recording. The quality of DIC images is considerably better in tissue slices prepared from relatively young animals, possibly due to a lesser degree of myelination (Dodt and Zeiglgansberger, 1990). Therefore, the application of this technique on slices prepared from adult animals can be problematic. In addition, as already mentioned, tissue slices are less viable in the DIC recording chambers, particularly when recording at, or near, physiological temperatures. This may be because only the top surface of the tissue is perfused with oxygenated aCSF in DIC recording chambers as the tissue rests on a glass bottom that is required for DIC imaging. Nevertheless, DIC-video-enhanced microscopy has made possible experiments that previously could only be hypothesized, and technical improvements will no doubt soon overcome some of these methodological limitations.

Many investigators have employed the whole-cell patch-clamp technique to study the effects of ethanol on synaptic transmission in brain slices. This recording method was used in the first demonstration that ethanol significantly inhibited the currents underlying NMDA receptor-mediated synaptic transmission in the mammalian CNS (Morrisett and Swartzwelder, 1993). Similarly, we used this technique in the first demonstration that ethanol could potentiate pharmacologically isolated GABA_A receptor-gated synaptic currents in rat hippocampal neurons (Weiner et al., 1994). Recently, we have used the patch-clamp technique in brain slices to uncover novel presynaptic effects of ethanol at GABAergic (Crowder et al., 2000; Ariwodola et al., 2001) and glutamatergic (Crowder and Weiner, 2001) synapses in the rat hippocampus and nucleus accumbens. We have also used this technique to characterize the acute effects of ethanol on excitatory and inhibitory synaptic transmission in monkey hippocampal slices (Ariwodola et al., 2000). Many other investigators continue to use these recording techniques to study the acute effects of ethanol on synaptic transmission (Soldo et al., 1998; Eggers et al., 2000; Poelchen et al., 2000) as well as the synaptic consequences of chronic ethanol exposure and withdrawal (Thomas et al., 1998b).

8.4 DISTINGUISHING BETWEEN PRESYNAPTIC AND POSTSYNAPTIC MECHANISMS OF ACTION OF ETHANOL

As already discussed, ethanol interacts with a number of targets within the central nervous system that participate, in a variety of ways, in synaptic transmission. One of the major advantages of brain slice preparations is that they retain intact synapses that can be triggered by electrical stimulation (Dingledine et al., 1980). This feature makes brain slices an ideal model system to elucidate the complex effects of ethanol on synaptic transmission. However, because both pre- and postsynaptic changes can

contribute to any observed effects of ethanol, identifying the specific mechanisms underlying modulatory effects of ethanol on synaptic transmission in brain slices can be difficult. For example, many studies have demonstrated that, at least under some conditions, ethanol enhances GABA_A receptor-mediated synaptic transmission in the rat hippocampal CA1 region (Weiner et al., 1994; Wan et al., 1996; Weiner et al., 1997a,b; Poelchen et al., 2000). However, despite much effort, the synaptic mechanism(s) underlying this effect have yet to be conclusively resolved. The quest to identify the specific loci that mediate various forms of synaptic plasticity has also proven to be a major challenge to slice electrophysiologists (e.g., Manabe et al., 1993; Nicoll and Malenka, 1999). A variety of methodological approaches have been developed to distinguish between pre- and postsynaptic sites of synaptic modulation. Three of the most common methods will be discussed and the advantages and disadvantages of each will be reviewed.

8.4.1 FOCAL APPLICATION OF AGONIST

One of the most straightforward approaches to delineate the locus of a synaptic modulation is to activate the postsynaptic ion channels directly by exogenous application of an appropriate agonist. The effect of the synaptic modulator can then be tested on these agonist-evoked responses. Since these agonist-evoked responses are generally devoid of any presynaptic influences, any observed effects of the modulator can be taken as evidence of a postsynaptic mechanism of action. This approach is frequently used in the study of recombinant ligand-gated ion channels expressed in cell lines or when studying the pharmacology of native ion channels in cells grown in culture. Methods for such preparations are described in Chapter 7 in this volume and typically involve rapid exchange of the solution bathing the cell being recorded. Rapid agonist application is necessary as most ligand-gated ion channels that underlie excitatory and inhibitory synaptic transmission undergo rapid desensitization when exposed to an agonist for prolonged intervals. While it is relatively straightforward to effect rapid solution exchanges across an isolated or cultured cell, such rapid bath exchanges are not generally possible in brain slice preparations. Therefore, alternative approaches have been developed that permit localized application of receptor agonists directly onto individual neurons in tissue slices. These methods involve filling a glass microelectrode, similar in shape and size to a patch electrode, with a receptor agonist of choice (e.g., GABA or glutamate). The electrode is then placed in proximity to the cell being recorded and agonist is released for brief intervals (usually 1–10 ms) using current injection (iontophoresis) or a brief pressure application.

Focal agonist application has been used in a number of brain slice electrophysiological studies to demonstrate that inhibitory effects of ethanol on NMDA- (Proctor et al., 1992) and kainate receptor-evoked EPSCs (Weiner et al., 1999) are mediated predominantly via postsynaptic mechanisms. These methods have also been used to determine the synaptic loci that mediate potentiation by ethanol of GABA_A receptor-mediated synaptic inhibition in rat hippocampal neurons. Initial studies reported that ethanol had no effect on GABA-evoked responses in hippocampal neurons (Proctor et al., 1992), even under conditions where ethanol did enhance GABA_A IPSP/Cs

(Wan et al., 1996). We have previously shown that ethanol enhances somatic GABA_A IPSCs to a greater extent than dendritic GABA_A synaptic responses (Weiner et al., 1997a). Therefore, in preliminary experiments, we used DIC-video enhanced microscopy to directly test the ethanol sensitivity of currents evoked by focal application of GABA directly onto the soma of CA1 pyramidal cells. In these experiments, 80 mM ethanol, which produced significant potentiation of somatic GABA_A IPSCs, had no effect on somatically evoked GABA currents. In contrast, GABA-evoked currents were potentiated by the GABA_A receptor modulator flunitrazepam and were completely abolished by the GABA_A receptor antagonist bicuculline methiodide (Figure 8.8). These experiments suggest that ethanol may not act solely via a postsynaptic mechanism. However, these negative results also highlight some of the limitations of this method. Even when carried out under direct visual guidance, it is not possible to precisely control the concentration of agonist that actually reaches the cell. This may be problematic when testing the effects of competitive agonists like ethanol that have been shown, using other methods, to potentiate GABA_A receptor function only at low GABA concentrations. Furthermore, it is not possible to ensure that the receptors being activated by focal agonist application are actually the same as those underlying the synaptic responses. Differences have been noted between the physiological and pharmacological properties of synaptic and extrasynaptic GABA_A receptors (Brickley et al., 1999; Bai et al., 2001). For these reasons, negative results obtained with focal agonist application in slices may be difficult to interpret.

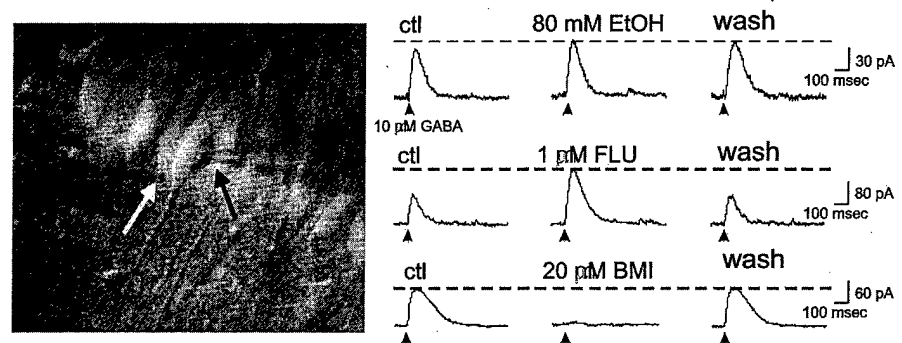


FIGURE 8.8 Ethanol has no effect on currents evoked by focal application of GABA in rat hippocampal CA1 pyramidal neurons. Photograph is a video-enhanced DIC image of the CA1 pyramidal layer of 300 μm rat hippocampal slice. Illustrated are the position of a patch-clamp recording electrode (right) sealed onto a CA1 pyramidal neuron and a pressure ejection electrode (left) filled with 10 μM GABA. Traces to the right of the photograph are averages of 3–5 GABA evoked currents recorded prior to, during, and after bath application of ethanol (EtOH), flunitrazepam (FLU), and bicuculline methiodide (BMI). Note that 1 μM FLU potentiates and 20 μM BMI completely inhibits GABA-evoked currents. However, 80 mM EtOH has no effect on these GABA-mediated responses.

8.4.2 PAIRED-PULSE FACILITATION RATIO

Because of the limitations associated with focal application of agonist in brain slices, a number of methods have been devised to evaluate the synaptic locus of a drug's action that rely solely on evaluating the effect(s) of the modulator on synaptic responses. One commonly employed method is to examine the effects of the modulator on paired-pulse facilitation (PPF), a form of short-term synaptic plasticity that is present at many synapses in the mammalian central nervous system (McNaughton, 1982; Manabe et al., 1993). PPF is induced by pairing two stimuli, typically with an interstimulus interval of 5–50 ms. This pairing results in a facilitation of the second response relative to the first that is thought to result from an increase in the probability of neurotransmitter release (Zucker, 1989). The ratio of Peak 2 to Peak 1 has been shown to be sensitive to a broad range of presynaptic experimental manipulations that alter the probability of neurotransmitter release (e.g., changing extracellular Ca^{++} concentration). However, this ratio is generally unaffected by postsynaptic manipulations, such as administration of a submaximal concentration of an antagonist of the synaptic receptor being studied (Creager et al., 1980; Dunwiddie and Haas, 1985; Manabe et al., 1993; Brundage and Dunwiddie, 1996).

The advantages of PPF in elucidating the synaptic locus of a drug's action are that it is relatively simple to carry out and has been subjected to extensive empirical validation. Moreover, this method can be employed with either single cell recordings or extracellular measures of synaptic transmission. In fact, PPF has even been used to probe the synaptic mechanisms underlying long-term synaptic plasticity in freely moving rats (Li et al., 2000). The main disadvantage of this method is that presynaptic manipulations that do not arise as a result of a change in release probability may not affect the PPF ratio, even though they are presynaptic in origin. For example, increasing the stimulation intensity used to evoke synaptic responses, a presynaptic manipulation, will increase the magnitude of these responses by recruiting more fibers but will not change the PPF ratio (Manabe et al., 1993).

Nevertheless, because many neuromodulators that act presynaptically seem to do so by changing release probability, PPF is still a commonly employed electrophysiological assay. For example, several recent studies have used PPF to support the hypothesis that cannabinoids reduce excitatory synaptic transmission via a presynaptic reduction in transmitter release at glutamatergic synapses (Gerdeman and Lovinger, 2001; Hoffman and Lupica, 2001; Huang et al., 2001). Several studies have also used PPF to characterize the synaptic mechanisms associated with ethanol exposure. Tan and colleagues (1990) showed an increase in PPF at glutamatergic synapses in the hippocampal CA1 region of rats that had been exposed to prenatal ethanol, consistent with a reduction in the probability of glutamate release at these synapses. Nelson and co-workers (1999) used PPF, along with a number of other measures of synaptic transmission, to elucidate the synaptic loci associated with the complex neuroadaptive changes associated with chronic ethanol exposure. In their study, rats were exposed to chronic intermittent ethanol for 2 weeks. One of their many interesting findings was that chronic ethanol exposure resulted in a significant reduction in somatic and dendritic excitatory synaptic responses in the hippocampal CA1 region when recorded in the continued presence of ethanol or within an hour of ethanol withdrawal. In contrast,

PPF was not significantly affected by this ethanol treatment, suggesting that a presynaptic reduction in the probability of glutamate release was unlikely to account for the reduction in glutamatergic neurotransmission associated with chronic ethanol exposure. Interestingly, although acute exposure to ethanol had no effect on PPF of the somatic population spike in slices recorded from control animals, ethanol did increase PPF in slices recorded from chronically treated subjects, revealing perhaps, that there are some changes in presynaptic function that occur following chronic intermittent ethanol exposure.

8.4.3 MINIATURE SYNAPTIC CURRENTS

As already described, synaptic responses can be triggered in acutely prepared brain slices by electrical stimulation of presynaptic afferents. Another feature of brain slice preparations is that synaptic responses can often be detected in the absence of external stimulation. With the enhanced signal-to-noise ratio afforded by whole-cell patch-clamp recording, spontaneously occurring excitatory and inhibitory synaptic currents can often be detected in whole-cell patch-clamp recordings. In some cases, these spontaneous synaptic currents arise from the firing of presynaptic cells within the slice whose afferent projections impinge upon the cell being recorded. However, synaptic responses can also result from the random fusion of neurotransmitter vesicles at presynaptic terminals. These latter responses, termed miniature postsynaptic currents, persist in the presence of drugs that block action potentials, like tetrodotoxin. Since these miniature synaptic currents typically reflect the activation of individual synapses, or at most, the synaptic contacts of a single presynaptic cell, these responses tend to be much smaller than electrically evoked synaptic responses. However, these spontaneous events are a powerful tool to characterize the mechanisms underlying the effects of synaptic modulators. Under most experimental conditions, the frequency of these miniature synaptic currents is extremely sensitive to presynaptic manipulations, thus providing an excellent index of presynaptic function. In contrast, the kinetics of the miniature synaptic currents are generally only altered by postsynaptic manipulations. Not surprisingly, miniature synaptic currents have been used extensively in many physiological and pharmacological studies of synaptic transmission (Mody et al., 1994). The main disadvantage of this method is that miniature synaptic currents can be very small, sometimes making it problematic to distinguish real synaptic events from the baseline noise. Therefore, care must be taken to ensure that any changes in the amplitude of miniature synaptic events do not result in an artifactual increase in their frequency, as more events may simply be detected from the baseline noise. Excellent software programs are available that facilitate event detection and can significantly reduce these problems (e.g., Mini-Analysis, Synptosoft Inc, Decatur, GA).

Because the application of the whole-cell patch-clamp technique in brain slices has been in common use for less than 10 years, relatively few studies have employed the analysis of miniature synaptic currents to characterize possible presynaptic effects of ethanol. One recent study used these methods, along with several other approaches, to demonstrate that ethanol potentiation of glycinergic synaptic transmission in rat spinal cord slices involves both pre- and postsynaptic mechanisms

(Eggers et al., 2000). These authors noted that ethanol significantly increased the frequency and amplitude of TTX-resistant miniature glycinergic currents in hypoglossal motoneurons in spinal cord slices (Figure 8.9). Additional experiments, including an analysis of the effects of ethanol on currents evoked by focal application of glycine directly onto HM neurons, demonstrated that ethanol acts both pre- and postsynaptically to facilitate glycinergic synaptic transmission in the rat spinal cord. Interestingly, the postsynaptic ethanol sensitivity of these synapses changed during development, with glycine-evoked currents recorded from neonatal rats (P1–3) being significantly less sensitive to ethanol than those recorded from juveniles (P9–13). In contrast, the presynaptic effects of ethanol at these synapses were not developmentally regulated.

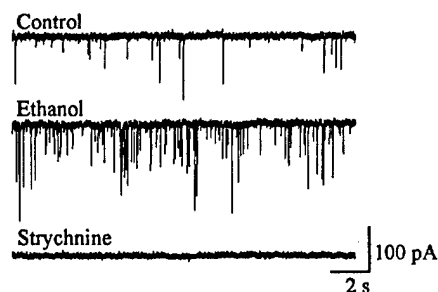


FIGURE 8.9 Glycinergic miniature inhibitory postsynaptic currents (mIPSCs) in hypoglossal motoneurons (HMs) are enhanced by ethanol and blocked by strychnine. Representative traces from a voltage-clamp recording of glycinergic mIPSCs in a HM from a juvenile rat [*postnatal day 9 (P9)*] are shown. Action potentials and nonglycinergic currents were blocked by tetrodotoxin (TTX), Cd^{++} , 6,7-dinitro-quinoxaline (DNQX), D(–)-2-amino-5-phosphonopentanoic acid (APV), and bicuculline methiodide (BMI). In this cell, adding 100 mM ethanol to the bath increased the average mIPSC amplitude by 32% and decreased the interval between successive mIPSCs by 58% (distributions significantly different by Kolmogorov-Smirnov test, $P < 0.001$). Glycinergic mIPSCs were blocked by strychnine (2 μM). (Reproduced with permission from Eggers et al., 2000.)

A number of additional methods are often employed to tease out the mechanisms underlying the effects of synaptic modulators. Measuring the frequency of failures in transmission in response to minimal stimulation of an individual synapse provides an alternative method to assess changes in release probability (e.g., Nicoll and Malenka, 1999; Cossart et al, 2001). In addition, methods have been developed to differentiate between pre- and postsynaptic mechanisms that take advantage of the quantal nature of synaptic transmission. These methods involve a statistical analysis of the variance in the amplitude of a synaptic response evoked repeatedly in the absence and presence of a synaptic modulator (Clements, 1990). In theory, the amplitude of an individual synaptic response will fluctuate between a finite numbers of equally spaced quantal levels (Fatt and Katz, 1951). Empirical studies have demonstrated that manipulations that are purely presynaptic will not affect the individual quantal levels but will alter their probabilities of occurrence. In contrast, postsynaptic manipulations may alter the quantal amplitudes but will not affect their relative probabilities of occurrence.

While empirical studies have provided some validation for all of the methods discussed, no single approach is likely to provide unequivocal proof of a synaptic mechanism of action. The best approach is to employ several different methods and, whenever possible, to empirically validate each method with known pre- and postsynaptic manipulations. This is particularly important when investigating a complex synaptic modulator like ethanol that may well have a variety of pre- and postsynaptic effects.

8.5 CONCLUSIONS AND FUTURE DIRECTIONS

The use of electrophysiological methods in acutely prepared brain slices has contributed immeasurably to our current understanding of how ethanol alters synaptic transmission in the mammalian CNS. The presence of functional synapses that are representative of the interneuronal connections observed *in vivo*, the ease of recording synaptic responses from individual neurons, and the ability to precisely control the time course and concentration of drug applications are just some of the features that make brain slice preparations a valuable tool for investigating the complex synaptic mechanisms underlying actions of ethanol.

A number of recent scientific and technical advances are providing even more applications for the use of brain slices in alcohol research. For example, over the last several years, a number of transgenic mouse models have been developed that display marked differences in their preference for or sensitivity to ethanol. Interestingly, many of the targeted genes are either involved in neurotransmitter synthesis (e.g., Weinshenker et al., 2000), encode for neurotransmitter receptors (e.g., Hall et al., 2001) or for signaling molecules that regulate the activity of synaptic ion channels (e.g., Miyakawa et al., 1997; Hodge et al., 1999; Thiele et al., 2000). It will be interesting in the coming years to delineate the synaptic correlates that underlie the behavioral differences in ethanol sensitivity in these various transgenic models using electrophysiological techniques in brain slices.

Another important technical advance has been the development of methods for culturing brain slices (Gahwiler, 1981). In this technique, brain slices are prepared from rat pups and grown for several weeks in culture media. These brain slice explant cultures maintain the cytoarchitectural organization of the tissue of origin and flatten out into layers of only 1-3 cell diameters, providing excellent visualization thus facilitating the use of patch-clamp and optical imaging techniques. Moreover, since these slices remain viable for several weeks, they provide an ideal model system for the investigation of the synaptic consequences associated with chronic alcohol exposure and withdrawal. Several studies have already employed slice culture methods to demonstrate upregulation of NMDA receptor function during alcohol withdrawal (Thomas et al., 1998a) and the possible cytotoxic sequelae associated with this upregulation in excitatory synaptic transmission (Prendergast et al., 2000; Thomas and Morrisett, 2000).

Finally, electrophysiological recording methods are but one of many experimental techniques that can be applied in brain slices. High resolution imaging of intracellular calcium levels (Conner et al., 1994) and electrochemical detection of neurotransmitters such as dopamine (Jones et al., 1999) are just two examples of

other methods that can be used in brain slices to provide additional insight into the physiology and pharmacology of synaptic transmission. Technical advances are rapidly making it possible to apply these, along with many other methods (e.g., RT-PCR, gene arrays), in concert with electrophysiological recordings of synaptic transmission in brain slices. Such multidisciplinary studies will likely yield important new insights into the complex mechanisms underlying the acute and chronic effects of ethanol.

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Ionotropic Glutamate Receptor Subunit Expression in the Rat Hippocampus: Lack of an Effect of a Long-Term Ethanol Exposure Paradigm

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Background: Studies have shown that acute ethanol exposure inhibits ionotropic glutamate receptor function and that long-term ethanol exposure results in maladaptive increases in the expression of some of these receptors in neurons. It has been postulated that these changes, when unopposed by ethanol, contribute, in part, to the hyperexcitability associated with ethanol withdrawal. In this study, we compared the effect of long-term ethanol exposure on the hippocampal expression levels of subunits belonging to the three families of ionotropic glutamate receptors.

Methods: Adult male Sprague-Dawley rats were fed an ethanol-containing diet for 16 days. This diet contained 0% ethanol on days 1 and 2, 3% on days 3 and 4, 5% on days 5 to 7, and 6.7% on days 8 to 16. Control rats received an equivalent amount of an isocaloric diet without ethanol. Rats were killed on day 16 at the peak of ethanol consumption. Hippocampal homogenates were prepared by sonication and analyzed by Western immunoblotting techniques. On a separate group of rats, we measured withdrawal scores and audiogenic seizures on day 17.

Results: Ethanol-exposed rats had significantly higher withdrawal scores, and a significantly higher percentage of them developed audiogenic seizures; this indicates that the 16-day ethanol diet induces ethanol dependence. Unexpectedly, we found that expression of NR1 (including the expression of NR1 subunits containing the N1, C1, and C2 inserts), NR2A, NR2B, NR2C, GluR1, GluR2/3, GluR5, GluR6/7, and KA2 subunits was not altered in hippocampal homogenates from ethanol-exposed rats.

Conclusions: These results indicate that maladaptive changes in the hippocampal expression levels of ionotropic glutamate receptor subunits do not always occur in ethanol-dependent rats. Consequently, other mechanisms must mediate the hyperexcitability state associated with ethanol withdrawal in these animals.

Key Words: Chronic, Alcohol, NMDA, AMPA, Kainate.

THE SUPERFAMILY OF glutamate-gated ion channels mediates the majority of excitatory transmission in the mammalian central nervous system and is composed of the NMDA, AMPA, and kainate receptors. Multiple polypeptide subunits have been identified for each of these glutamate receptor subfamilies. Six NMDA receptor (NMDA-R) subunits (NR1, NR2A, NR2B, NR2C, NR2D, and NR3A), four AMPA receptor subunits (GluR1, GluR2, GluR3, and GluR4), and five kainate receptor subunits (GluR5, GluR6, GluR7, KA1, and KA2) have been characterized to date [for a review, see Ozawa et al.

(1998)]. Three exons of the NR1 subunit mRNA can be differentially spliced. They encode 21 to 38 amino acid sequences located in the N-terminus domain (termed the N1 cassette) or the C-terminus domain (termed the C1 and C2 cassettes) [for a review, see Zukin and Bennett (1995)]. These NMDA-R subunits and NR1 alternatively spliced variants can form channels with diverse subunit compositions and functional properties. The expression levels of these subunits are precisely regulated by complex signal transduction processes, and changes in ionotropic glutamate receptor subunit expression levels have been shown to have important roles, not only in normal development and synaptic plasticity, but also in pathophysiological conditions.

Long-term exposure to ethanol has been shown to induce maladaptive changes in the protein levels of glutamate-gated ion channel subunits [reviewed in Valenzuela and Harris (1997)]. Western blot studies detected increases in NR1 protein levels in brain synaptosomes (Chen et al., 1997) and in hippocampal homogenates (Devaud and Morrow, 1999; Trevisan et al., 1994) from rats chronically exposed to ethanol. Another study found increases in NR1, NR2A, and NR2B protein levels in homogenates from the

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cortex and hippocampus of rats exposed to intragastric infusions of ethanol for 6 days (Kalluri et al., 1998). An increase in hippocampal NR1 and NR2A subunit levels was reported by Snell et al. (1996) in mice exposed to an ethanol-containing liquid diet for 2 weeks. Taken together, the results of these studies with chronically ethanol-exposed animals suggest that NMDA-R subunit expression is altered by long-term ethanol exposure. It has been postulated that these maladaptive changes in NMDA-R subunit expression, when unopposed by ethanol, contribute, in part, to the neurotransmission imbalance associated with the ethanol withdrawal syndrome.

Much less is known about the effects of long-term ethanol exposure of animals on the expression of non-NMDA-R subunits. One study reported that a 12-week exposure to ethanol did not change the levels of GluR1 and GluR2 subunits in the rat hippocampus (Trevisan et al., 1994). However, the effect of long-term ethanol exposure on the expression of other non-NMDA-R subunits in the mammalian brain remains an open question. In this study, we examined the effects of a 16-day ethanol diet on the expression of subunits belonging to the NMDA, AMPA, and kainate families of glutamate receptors. We concentrated our studies on the rat hippocampus because ionotropic glutamate receptors have been shown to have important roles in seizure generation in this brain region (Ben-Ari and Cossart, 2000; Kullmann et al., 2000).

METHODS

Chronic Ethanol Exposure Paradigm

All animal experiments were authorized by the University of New Mexico Health Sciences Center-Institutional Animal Care and Use Committee and performed in accordance with its guidelines. Rats were maintained in the Animal Resource Facilities at the University of New Mexico School of Medicine and allowed to acclimate to the rat room environment for at least 1 week before initiation of the diet described below. Adult male Sprague-Dawley rats weighing approximately 250 g were individually housed in a room maintained at 22°C on an 8-hr dim light/16-hr dark cycle (lights on from 0900 to 1700 hr). Rats received a Bioserv (Frenchtown, NJ) chocolate-flavored liquid diet that was based on the Lieber-DeCarli formulation (Lieber and DeCarli, 1982). This liquid diet provides 1 kcal/ml. A group of rats (ethanol-treated group) was offered 80 ml of diet containing 0% v/v (days 1–2), 3% v/v (days 3–4), 5% v/v (days 5–7), and 6.7% v/v ethanol (days 8–16). Rats consumed approximately 80 ml/day during days 1 to 4, approximately 60 to 70 ml/day during days 5 through 7, and approximately 40 to 65 ml/day during days 8 to 16. A control group of rats was given equivalent amounts of the liquid diet without ethanol; their diet was made isocaloric to the ethanol-containing diet by the addition of maltose-dextrins. The liquid diet was available to the rats during the 16-hr dark cycle (starting at 1700 hr), and water bottles were removed from the cages during this period. The liquid diet tubes were removed at 0900 hr each morning and the water bottles returned to the cages. Tubes were removed in the morning because rats are nocturnal animals that do not consume significant amounts of this liquid diet during the light cycle (D.D. Savage et al., unpublished data, 2001). Rats had free access to water during the light hours to prevent dehydration. Diet consumption patterns were checked daily. We did not detect statistically significant differences in body weight between the ethanol and control groups (Fig. 1). To confirm that this diet induced ethanol dependence, we determined baseline withdrawal scores on a separate group of rats by using the scale described by

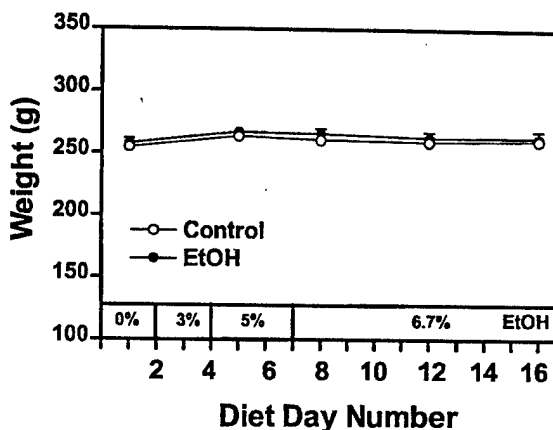


Fig. 1. Shown is the weight of chronically ethanol (EtOH)-treated rats and control rats. Values were not found to be statistically different by two-way ANOVA ($p > 0.3$; $n = 8$ rats per group).

Lal et al. (1988) (Table 1). In this group of rats, we also measured audiogenic seizures. Individual rats were placed in a circular chamber (40 cm in diameter \times 60 cm high). A sound stimulus (108 dB relative to 2×10^{-4} dynes/cm²) was generated by an electric bell mounted on the ceiling of the chamber. The sound stimulus was initiated 15 sec after the rat was placed into the chamber and was continued until the onset of convulsion or for a maximum of 90 sec if no convulsion was observed. Convulsions in these rats were characterized by a running phase, followed by clonic and, in most cases, tonic convulsions. Data are given as the percentage of animals tested per diet group that developed seizures.

Rats were killed by rapid decapitation without anesthesia. They were euthanized at the peak time (2200–2330 hr) of ethanol consumption on day 16 of the ethanol treatment. After death, blood was obtained from the heart into ethylenediaminetetraacetic acid-containing tubes, and blood ethanol levels were measured with a kit based on the activity of the enzyme alcohol dehydrogenase. At this time point, blood ethanol levels ranged from 20 to 50 mM. After decapitation, brains were rapidly removed and hippocampi dissected out on ice. Hippocampi were homogenized by sonication in phosphate-buffered saline plus a protease inhibitor cocktail (P-8340, Sigma Chemical Co., St. Louis, MO). The protein content of the homogenates was determined by Lowry assay. Samples were diluted to 2

Table 1. Baseline Withdrawal Scores Obtained on Day 17 at 1530 hr (see "Methods" for more details) on a Separate Group of Rats

Parameter scored	Group	
	Control	Ethanol
General activity	0.5 \pm 0.2	0.7 \pm 0.1
Shakes, jerks, and twitching	0.0	0.3 \pm 0.3
Head tremor	0.5 \pm 0.2	1.0 \pm 0.3
Vocalization	0.6 \pm 0.3	2.1 \pm 0.2*
Avoidance	1.0 \pm 0.2	1.7 \pm 0.3*
Rigidity of axial muscles by palpation	1.2 \pm 0.1	1.7 \pm 0.1*
Tail tremor	1.2 \pm 0.1	1.5 \pm 0.2
General tremor	1.0 \pm 0.0	1.8 \pm 0.1*
Motor task	1.1 \pm 0.1	2.1 \pm 0.2*
Bracing posture	1.1 \pm 0.1	1.5 \pm 0.2*
Convulsions	1.0 \pm 0.0	1.1 \pm 0.1
Tail-lifting tremor	0.6 \pm 0.3	2.2 \pm 0.2*
Grooming	0.6 \pm 0.2	1.8 \pm 0.3*
Average scores	0.8 \pm 0.1	1.5 \pm 0.1**
Audiogenic seizures	0%	80%

Scores were obtained as described by Lal et al. (1988). Values represent mean \pm SEM of five rats per treatment group.

* $p < 0.05$ and ** $p < 0.01$ by t test.

Shown at the end of the table is the percentage of these rats that developed audiogenic seizures.

to 4 mg/ml with phosphate-buffered saline, mixed with 2× SDS-PAGE sample buffer, boiled for 2 min, and stored in aliquots at -20°C .

Western Immunoblotting

Samples (10–30 μg of total protein per lane) were separated on 7.5% polyacrylamide minigels and electrotransferred to nitrocellulose membranes. Nonspecific binding of antibodies to nitrocellulose membranes was prevented by blocking with a solution containing 10% nonfat dry milk and 0.4% Tween-20 (ICI Americas, Inc., Wilmington, DE), plus 0.01% sodium azide. Blots were analyzed with a chemiluminescence assay kit by following the manufacturer's instructions (Roche Biochemicals, Indianapolis, IN). Membranes were then probed with rabbit anti-NR1-N1 insert, anti-NR1-C1 insert, anti-NR1-C2 insert, anti-NR2A, anti-NR2B, and anti-NR2C antibodies (all produced, purified, and characterized in MDB's laboratory). Membranes were also probed with rabbit anti-GluR6/7, anti-KA2, anti-GluR1, and anti-GluR2/3 antibodies from Upstate Biotechnology (Lake Placid, NY) and with goat anti-GluR5 or anti-NR1 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-rabbit and anti-goat immunoglobulin G-peroxidase conjugates were obtained from Roche Biochemicals. Densitometric analyses of Western blot chemiluminescence x-ray films were performed by using an Image-Pro® Plus image analysis system (Media Cybernetics, L.P., Silver Spring, MD). In all cases, a protein standard curve was generated from a single batch of hippocampal tissue homogenate kept at -20°C . This standard curve was included in the same membrane (by using 15-well combs) as samples from control and chronic ethanol-treated animals. By using linear regression analysis, this curve was used to calculate relative units of subunit protein concentrations in samples from control and ethanol-exposed animals (Chandler et al., 1999). Tubulin levels (anti- β -tubulin monoclonal antibody from Sigma) were measured in all samples, and relative units of subunit protein were divided by the relative units of tubulin in each sample to normalize for differences in gel loading. Tubulin levels were unaffected by the long-term ethanol exposure paradigm used in this study (control, 17.2 ± 1.2 , and ethanol-exposed, 16.7 ± 1.2 densitometry arbitrary units; $n = 8$).

RESULTS

To confirm that this diet induced ethanol dependence, we determined withdrawal scores by using a modified version of a previously described scale (Table 1) (Lal et al., 1988). These determinations were performed with a separate group of animals that were not used for Western blot analyses. The parameters listed in Table 1 were scored exactly as described by Lal et al. (1988) by two observers that were blind to the treatment group assignment of each rat. On day 17 at 1530 hr, we observed statistically significant differences between control and ethanol-treated rats in several of the scored parameters, including vocalizations, general tremor, motor task, tail-lifting tremor, and grooming (Table 1). A trend toward an increase was also found in other parameters, such as avoidance, rigidity of axial muscles by palpation, head tremor, and bracing posture (Table 1). Average scores were significantly increased in the ethanol-treated animals (Table 1). We also assessed the development of audiogenic seizures in this group of animals. None of the five rats from the control group developed audiogenic seizures (Table 1). Conversely, four of five animals in the chronically ethanol-exposed group developed audiogenic seizures (Table 1). Taken together, these results clearly indicate that our diet-exposure paradigm induces ethanol dependence. It should be emphasized,

however, that we performed these studies only to establish that the ethanol-exposure paradigm produced ethanol dependence in our hands and that our studies of the effect of ethanol on hippocampal glutamate receptor expression were not aimed at determining the mechanism of ethanol withdrawal-induced audiogenic seizures; the neuronal network for the generation of these seizures is primarily contained in certain brainstem structures [reviewed in Faingold et al. (1998)].

Western blot analyses were performed to assess expression of NMDA, AMPA, and kainate hippocampal receptor subunits during the last day of the diet-exposure paradigm. For these studies, rats were killed on day 16 at the peak of ethanol consumption. We initially studied expression of NMDA-R subunits and did not detect a significant effect of chronic ethanol exposure on NR1, NR2A, NR2B, or NR2C subunit levels (Fig. 2). Moreover, we did not detect significant effects of long-term ethanol exposure on expression levels of NR1 subunits containing the N1, C1, or C2 cassettes (Fig. 3).

We also measured expression of non-NMDA-R subunits. We assessed expression of AMPA receptor subunits with anti-GluR1 and anti-GluR2/3 antibodies. We did not detect significant effects of chronic ethanol exposure on the expression levels of these subunits in hippocampal homogenates (Fig. 4). Expression levels of kainate receptor subunits were determined with anti-GluR5, -GluR6/7, and -KA2 antibodies. As with NMDA and AMPA receptor

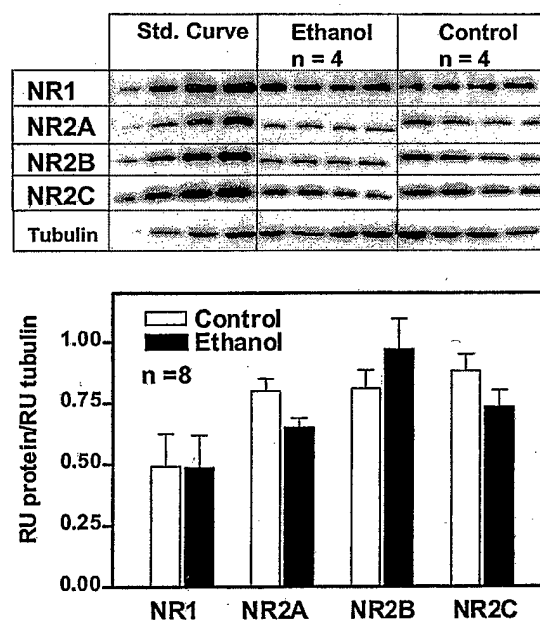


Fig. 2. Shown in the upper panel are samples of Western immunoblots of hippocampal homogenates from ethanol-treated and control rats that were probed with the indicated anti-NMDA receptor subunit antibodies. As illustrated, a standard curve was generated with a single batch of sample in all cases. Values from this standard curve were used to calculate relative units (RU) of protein concentration. Relative units of tubulin levels were also determined and used to normalize the results (see "Methods" for details). Shown in the lower panel is the summary of the results. Values were not found to be statistically different by two-way ANOVA ($p > 0.16$).

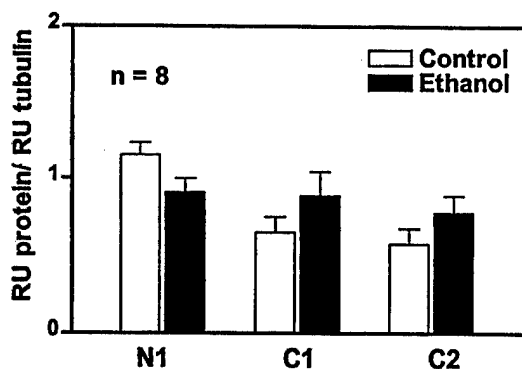
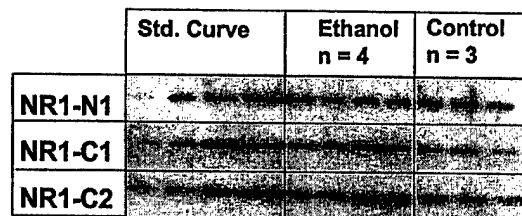


Fig. 3. Shown in the upper panel are samples of Western immunoblots of hippocampal homogenates from ethanol-treated and control rats that were probed with antibodies that recognize NR1 subunits containing the N1, C1, or C2 cassettes. See the legend for Fig. 2 for more details. Shown in the lower panel is the summary of the results. Values were not found to be statistically different by two-way ANOVA ($p > 0.5$). RU, relative unit.

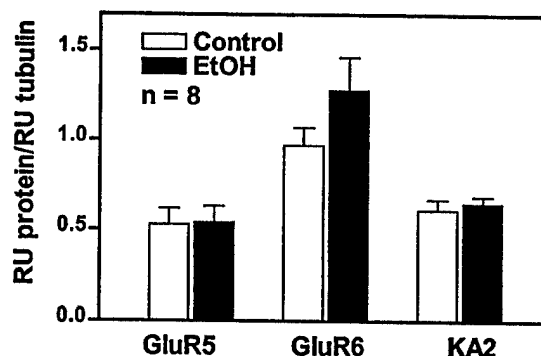
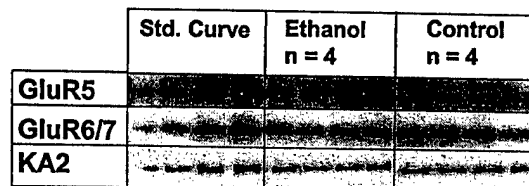


Fig. 5. Shown in the upper panel are samples of Western immunoblots of hippocampal homogenates from ethanol-treated and control rats that were probed with the indicated anti-kainate receptor subunit antibodies. See the legend for Fig. 2 for more details. Shown in the lower panel is the summary of the results. Values were not found to be statistically different by two-way ANOVA ($p > 0.4$). RU, relative unit.

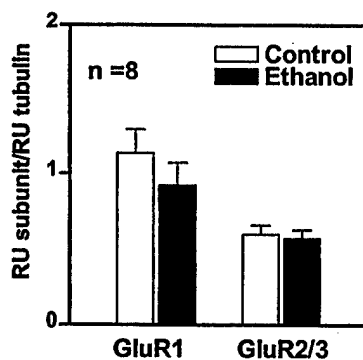
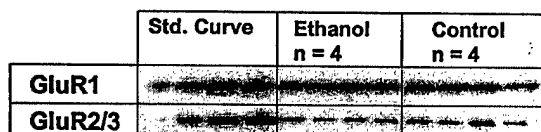


Fig. 4. Shown in the upper panel are samples of Western immunoblots of hippocampal homogenates from ethanol-treated and control rats that were probed with the indicated anti-AMPA receptor subunit antibodies. See the legend for Fig. 2 for more details. Shown in the lower panel is the summary of the results. Values were not found to be statistically different by two-way ANOVA ($p > 0.4$). RU, relative unit.

subunits, we did not detect alterations in the levels of kainate receptor subunit expression in the chronically ethanol-exposed animals (Fig. 5).

DISCUSSION

In this study we examined the effects of a 16-day ethanol-containing diet on the expression of receptor subunits be-

longing to all three families of glutamate-gated ion channels. We studied a separate group of rats the day after the end of this diet and determined, by assessing both withdrawal scores and the presence of audiogenic seizures, that it clearly results in the development of ethanol dependence. Unexpectedly, we did not find differences in NMDA-R subunit expression in the hippocampus of rats when assessed at the peak of ethanol consumption on day 16 of this diet. Our results are somewhat unexpected because other studies have reported changes in NMDA-R subunit expression after long-term ethanol exposure paradigms. For instance, a recent study by Devaud and Morrow (1999) found an approximately 20% increase in the levels of NR1 and NR2A subunit expression in hippocampal homogenates of male Sprague-Dawley rats exposed to a liquid diet that produced blood ethanol levels in the 35 to 50 mM range and that were killed while still on this diet. Kalluri et al. (1998) found an approximately 35% increase in the levels of NR1, NR2A, and NR2B in the hippocampus of male Sprague-Dawley rats that received 9 to 15 g/kg of ethanol per day by intragastric intubation three times a day for 6 days and that were killed 1 hr after the last dose of ethanol. Trevisan et al. (1994) detected an approximately 65% increase in NR1 immunoreactivity in the hippocampus of male Sprague-Dawley rats that were exposed to a 12-week ethanol-containing liquid diet. These rats had blood ethanol levels of 50 to 65 mM at the time of death. In contrast to these studies, Winkler et al. (1999) found that hippocampal NR1 subunit levels did not change in alcohol-preferring (AA) or alcohol-nonpreferring (ANA) rats exposed to an ethanol-containing diet, which produced maximum blood

ethanol levels of approximately 30 mM, for 30 days. In agreement with the study of Winkler et al., we did not find an effect of long-term ethanol on hippocampal ionotropic glutamate receptor expression; this indicates that changes in NMDA-R subunit expression do not always occur in rats chronically exposed to ethanol.

The effects of chronic ethanol exposure on the expression of alternatively spliced isoforms of the NR1 subunit have recently been studied, with emphasis on messenger RNA (mRNA) levels. Hardy et al. (1999) reported a decrease in the ratio of NR1 mRNA containing the segment encoding for the N1 insert in the cerebral cortex of male Wistar rats exposed to ethanol vapor for 16 days. These rats were killed before withdrawal and had blood ethanol levels of 50 to 100 mM. A more recent *in situ* hybridization study, also performed with male Wistar rats, detected a decrease in hippocampal mRNA levels of NR1 subunits containing the C2 (NR1-2) insert after administration of 9 to 15 g/kg/day of ethanol for 8 days via intragastric intubation (Darstein et al., 2000). In the study of Winkler et al. (1999) discussed previously, it was found that chronic ethanol exposure of AA and ANA rats did not affect mRNA levels for NR1-1 (contains the C1 and C2 cassettes), NR1-2 (contains the C2 cassette), NR1-3 (contains the C1 cassette), or NR1-4 (does not contain the C1 or C2 cassettes). It is interesting that Western blot analyses revealed that the signal obtained with an anti-NR1-3/1-4 antibody significantly increased in ethanol-exposed AA rats, but not in ANA rats (Winkler et al., 1999). In contrast to these studies, we found no effects of long-term ethanol exposure of male Sprague-Dawley rats on the protein expression levels of hippocampal NR1 subunits containing the N1, C1, and C2 inserts. However, it is difficult to compare our study with the work of Hardy et al. (1999) and Darstein et al. (2000) because these studies did not examine the protein expression levels of NR1 subunit splice variants. Moreover, it is also difficult to compare our results with those of Winkler et al. (1999) because they used antibodies that cannot distinguish among subunits containing the C1 cassette only (NR1-3) or subunits lacking the C1 and C2 cassettes (NR1-4). Therefore, it would be important to determine whether exposure to chronic exposure paradigms such as the one used by Winkler et al. (1999) produces changes in the protein levels of NR1 splice variants when assessed with more specific antibodies.

An important finding of our study is that we did not detect any differences in the expression of GluR1 and GluR2/3 subunits. These results are in agreement with those of Trevisan et al. (1994), who reported that a 12-week exposure to alcohol did not change hippocampal levels of either GluR1 or GluR2 subunits in the hippocampus of male Sprague-Dawley rats. We also evaluated expression of GluR5, GluR6/7, and KA2 kainate receptor subunits and found it to be unaltered after 16 days of ethanol exposure. To the best of our knowledge, this is the first study on the effects of long-term ethanol exposure of rats on kainate

receptor subunit expression. Therefore, it would also be important to corroborate our findings by using different ethanol-exposure paradigms than the one used in this study.

It is important to compare the findings of our study with those of some investigations on the long-term effects of ethanol exposure of cultured neurons. Ticku and collaborators have reported that exposure of cultured cortical neurons to 50 to 75 mM ethanol for 5 days results in an increase in [³H]MK-801 binding and also in up-regulation of NR1 and NR2B mRNA and protein subunit levels (Follesa and Ticku, 1995, 1996; Hu and Ticku, 1995; Hu et al., 1996; Kumari and Ticku, 1998). Chandler et al. (1997) reported that ethanol exposure (100 mM for 4 days) of cultured cortical neurons grown in the presence of 2 mM glutamine did not change [¹²⁵I]MK-801 binding or the expression levels of NR1, NR2A, and NR2B subunits. However, this treatment increased NMDA-stimulated nitric oxide production. A subsequent study by the same group of investigators, which was also performed with cultured cortical neurons exposed to 100 mM ethanol for 4 days, detected an increase in levels of NR1, NR2A, NR2B, GluR1, and GluR2/3 subunits, but not of GluR6/7 subunits (Chandler et al., 1999). It is important to note that these ethanol-induced changes in subunit expression were highly dependent on the culture conditions, because these were observed in cells grown in low (0.1 mM), but not high (2 mM), glutamine. Hoffman et al. (1995, 1996) reported a 47% increase in [³H]MK-801 binding and a 20% increase in NR1 and a 30% decrease in NR2A subunit levels in cultured cerebellar granule neurons exposed to 100 mM ethanol for 2 to 4 days. In contrast, Cebere et al. (1999) reported that exposure of cultured cerebellar granule neurons to 50 to 100 mM ethanol for 3 days did not change [³H]MK-801 binding or expression of NR1 splice variants and NR2A, NR2B, and NR2C subunits. More recently, Kumari (2001) found that exposure of cultured cortical neurons to 50 mM ethanol for 5 days induced a decrease in the mRNA and protein levels of splice variants containing the N1 cassette (NR1-3b and NR1-4b) and an increase in the protein expression levels of splice variants lacking this cassette (NR1-3a and NR1-4a). As in the case of the animal studies mentioned previously, the results of these studies clearly indicate that the effects of long-term ethanol exposure of cultured neurons on ionotropic glutamate receptor subunit expression are also variable and highly dependent on the experimental conditions used by different investigators. However, an important conclusion that can be derived from these *in vitro* studies is that chronic ethanol exposure does not always result in compensatory changes in ionotropic glutamate receptor subunit expression; this is in agreement with the findings of our study.

In conclusion, we found that exposure of rats to a 16-day ethanol exposure paradigm, which clearly results in the development of ethanol dependence, is not associated with changes in the hippocampal expression of subunits belong-

ing to all of the three major families of ionotropic glutamate receptors. Our findings are in agreement with those of Rudolph et al. (1997), who reported that robust changes in ligand binding to glutamate receptors in the adult rat brain do not occur with several chronic ethanol treatment protocols, including a liquid diet similar to the one used in this study. Therefore, maladaptive changes in brain ionotropic glutamate receptor levels do not underlie, in all cases, the neurobehavioral consequences of chronic ethanol exposure.

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Functional Characterization of Kainate Receptors in the Rat Nucleus Accumbens Core Region

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Crowder, Tara L. and Jeff L. Weiner. Functional characterization of kainate receptors in the rat nucleus accumbens core region. *J Neurophysiol* 88: 41–48, 2002; 10.1152/jn.00919.2001. The nucleus accumbens, a brain region involved in motivation, attention, and reward, receives substantial glutamatergic innervation from many limbic structures. This excitatory glutamatergic input plays an integral role in both normal and pathophysiological states. Despite the importance of glutamatergic transmission in the nucleus accumbens, the specific receptor subtypes that mediate glutamatergic signaling in this brain region have not been fully characterized. The current study sought to examine the possible role of the kainate subclass of glutamate receptor in the nucleus accumbens. Kainate receptors are relatively poorly understood members of the ionotropic glutamate receptor family and are highly expressed in the nucleus accumbens. Recent studies have highlighted a number of novel pre- and postsynaptic functions of kainate receptors in several other brain regions. Using the whole cell patch-clamp technique, we report the first demonstration of functional kainate receptors on neurons within the core region of the nucleus accumbens. In addition, we present evidence that activation of kainate receptors in this brain region inhibits excitatory synaptic transmission via a presynaptic mechanism.

INTRODUCTION

The nucleus accumbens (NAcc) is thought to serve as an interface between limbic and motor systems (Mogenson et al. 1980) and is involved in a number of processes including motivation (Mogenson et al. 1980), attention (Solomon and Staton 1982; van den Bos et al. 1991), and reward (Apicella et al. 1991; Colle and Wise 1988). Limbic innervation of the NAcc includes substantial glutamatergic input from the hippocampus, amygdala, and medial prefrontal cortex (Phillipson and Griffiths 1985). These excitatory inputs have been demonstrated to play an important role in the neurophysiology of the NAcc (Goto and O'Donnell 2001; O'Donnell and Grace 1995). Furthermore, it has been hypothesized that dysregulation of glutamatergic input to the NAcc may underlie the development of psychiatric disorders such as schizophrenia (O'Donnell and Grace 1998) and drug addiction (Wolf 1998). However, the receptor mechanisms responsible for the processing of glutamatergic input within the NAcc have not been fully characterized.

Glutamate, the primary excitatory neurotransmitter in the CNS, activates three major subclasses of ionotropic receptors:

N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and kainate (KA). Studies that have examined the function of glutamate receptors in the NAcc suggest that the excitatory effects of glutamate, *in vivo*, are primarily mediated by non-NMDA type (AMPA/KA) receptors (Hu and White 1996; Pennartz et al. 1991). Historically, it has been difficult to distinguish KA receptor (KA-R) from AMPA receptor (AMPA-R) function due to a lack of selective agonists and antagonists. The recent development of selective AMPA-R antagonists has resulted in a number of studies that have begun to unravel the functional role of KA-Rs within the mammalian CNS (for review see Ben-Ari and Cosart 2000; Frerking and Nicoll 2000). To date, KA-Rs have been shown to subserve a traditional postsynaptic role, gating synaptic excitation, in only a limited number of brain regions (Castillo et al. 1997; Frerking et al. 1998; Li and Rogawski 1998; Vignes and Collingridge 1997). However, KA-Rs have also been shown to function as presynaptic receptors at many synapses (Chittajallu et al. 1996; Chergui et al. 2000; Clarke et al. 1997; Frerking et al. 2001; Kamiya and Ozawa 1998, 2000; Kerchner et al. 2001; Rodriguez-Moreno et al. 1997; Schmitz et al. 2000, 2001; Vignes et al. 1998). Activation of these presynaptic KA-Rs potentially modulates both glutamate and γ -aminobutyric acid (GABA) release. Thus if functional KA-Rs are present in the NAcc, they may play an important role in regulating excitatory and inhibitory activity within this brain region.

The aim of the current study was to identify functional KA-Rs within the NAcc and to determine the role of KA-Rs in regulating excitatory activity within this nucleus. Using the whole cell patch-clamp technique, we demonstrate that functional KA-Rs are present on neurons within the NAcc core. These receptors can be activated by exogenous application of KA but do not contribute to excitatory postsynaptic currents (EPSCs) elicited by individual stimuli. Our data also suggest that activation of KA-Rs in the NAcc potentially inhibits glutamatergic synaptic transmission via a presynaptic mechanism.

METHODS

Slice preparation

Male Sprague-Dawley rats (20–40 days old) were anesthetized with halothane and killed by decapitation using a protocol approved

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by the ACUC of Wake Forest University School of Medicine. Coronal NAcc slices (400 μm) were prepared using a vibrating tissue slicer (Leica VT1000S; Vashaw Scientific, Atlanta, GA). Slices were then maintained at room temperature in oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 3.3 KCl, 2.4 MgCl_2 , 2.5 CaCl_2 , 1.2 KH_2PO_4 , 10 D-glucose, and 25.9 NaHCO_3 , saturated with 95% O_2 -5% CO_2 . During recordings, slices were perfused with oxygenated ACSF at a flow rate of 2 ml/min. All drugs were applied through the ACSF in known concentrations via calibrated syringe pumps (Razel; Stamford, CT).

Patch-clamp recordings

Methods for whole cell recordings were similar to those reported previously (Weiner et al. 1999). Briefly, electrodes were prepared from filamented borosilicate glass capillary tubes (ID: 0.86 mm) using a horizontal micropipette puller (Sutter P-97; Sutter, Novato, CA). Electrodes were filled with a recording solution containing (in mM) 130 K-gluconate, 10 KCl_2 , 5 N-(2,6-dimethyl-phenylcarbamoyl-methyl)-triethylammonium bromide (QX-314), 1 ethylene glycol-bis-(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 CaCl_2 , 2 Mg-ATP, 0.2 tris-GTP, and 10 HEPES (free acid). Whole cell patch-clamp recordings were made at room temperature from NAcc core neurons voltage clamped at -70 mV (for AMPA EPSCs) or -20 to -40 mV (for NMDA EPSCs). Recording electrodes were placed ventral to the anterior commissure within the core of the NAcc. Unless otherwise indicated, synaptic currents were evoked every 20 s by electrical stimulation (0.2 ms duration) of tissue adjacent to the recording electrode using a concentric bipolar stimulating electrode (FHC, Bowdoinham, ME). In one experiment, AMPA (10 μM) was applied directly to the soma of NAcc neurons using a Picospritzer III (General Valve, Fairfield, NJ). Recordings were acquired with an Axoclamp 2B amplifier, digitized (Digidata 1200B; Axon Instruments, Foster City, CA), and analyzed on- and off-line using an IBM compatible PC computer and pClamp 8.0 software (Axon Instruments, Foster City, CA).

Pharmacological isolation

Drugs used in the pharmacological isolation of evoked currents included the NMDA receptor antagonist DL-(+)-2-amino-5-phosphonovaleric acid (APV), the GABA_A receptor antagonist bicuculline methbromide (BIC), the AMPA/KA receptor antagonists 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[f]quinoxaline-7-sulfonamide (NBQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX); and the nonselective adenosine receptor antagonist theophylline (all from Sigma, St. Louis, MO). DNQX and NBQX were made up as stock solutions in dimethyl sulfoxide (DMSO; final total concentration of DMSO was $<0.05\%$). APV, BIC, and theophylline were made up as stock solutions in deionized water. The metabotropic glutamate receptor (mGluR) type I/II antagonist (RS)- α -methyl-4-carboxyphenylglycine (MCPG), the mGluR type III antagonist α -cyclopropyl-4-phosphonophenylglycine (CPPG), and the GABA_B receptor antagonist (SCH 50911) were also used (all from Tocris, Bristol, UK). MCPG was made up as a stock solution in 0.1 N NaOH and diluted in ACSF. Both CPPG and SCH 50911 were made up as stock solutions in deionized water.

Statistics

KA effects on the amplitude of EPSCs were defined as percent of control (predrug) values. Agonist-induced inward currents were defined as a difference from control holding current values (i.e., drug value - predrug value) expressed in picoamperes. Either one-way ANOVA or Student's *t*-tests were then used to analyze these data. When appropriate, post hoc analyses were performed using Dunnett's for comparing multiple groups to control, and Newman-Keuls for all pair-wise comparisons, with significance set at $P < 0.05$.

RESULTS

Functional kainate receptors are present on postsynaptic neurons in the NAcc

Although KA-R subunits, including GluR 6/7, and to a lesser extent KA2, have been shown to be expressed in both rodent (Bischoff et al. 1997) and primate (Charara et al. 1999; Kieval et al. 2001) ventral striatum, direct evidence of functional KA-Rs in this brain region is lacking. To test the hypothesis that KA-R subunits expressed in the ventral striatum form functional receptors on NAcc neurons, we measured the amplitude of inward currents evoked by bath application of KA (0.1–1 μM) in the presence of 50 μM APV and 20 μM BIC. Bath application of KA resulted in a concentration-dependent inward current in all cells tested (Fig. 1B). The inward currents evoked by the highest concentration of KA (1 μM) were not significantly attenuated by pretreating the slices with 1 μM NBQX (Fig. 1A). This concentration of NBQX has been shown to maximally block AMPA-Rs without having any effect on KA-Rs in mouse hippocampal slices (Bureau et al. 1999). To ensure that NBQX was also selective for AMPA-Rs over KA-Rs in the rat NAcc, we tested the effect of 1 μM NBQX on AMPA-induced inward currents. Bath application of 5 μM AMPA produced inward currents of similar amplitude to those induced by the application of 1 μM KA (Fig. 1A). Pretreatment of ventral striatal slices with 1 μM NBQX completely blocked AMPA-induced inward currents (Fig. 1A). Thus 1 μM NBQX selectively blocked AMPA-R, but not KA-R function in NAcc neurons. Although KA currents were not altered under conditions where AMPA-Rs were selectively blocked, these currents were completely inhibited by a saturating concentration of the nonselective AMPA/KA receptor antagonist, DNQX (80 μM ; Fig. 1A).

We next tested whether or not KA-Rs contributed to non-NMDA EPSCs in the NAcc core. Non-NMDA EPSCs were elicited by single pulse stimulation of glutamatergic afferents near the recorded neuron in the presence of 50 μM APV and 20 μM BIC. This stimulation protocol evoked a fast inward postsynaptic current in all cells. These non-NMDA EPSCs were completely inhibited by selectively blocking AMPA-Rs with 1 μM NBQX (Fig. 1C).

Kainate inhibits evoked EPSCs

We also sought to determine whether activation of KA-Rs in the NAcc core could alter excitatory synaptic transmission, as recently shown in the hippocampus (Chittajallu et al. 1996; Contractor et al. 2000; Frerking et al. 2001; Kamiya and Ozawa 1998, 2000; Schmitz et al. 2001; Vignes et al. 1998). This hypothesis was tested by evaluating the effect of bath application of KA on the amplitude of pharmacologically isolated AMPA-R- or NMDA-R-mediated EPSCs recorded from NAcc core neurons.

AMPA-R-mediated EPSCs were evoked every 20 s with individual stimuli in the presence of 50 μM APV and 20 μM BIC. A 7-min bath application of KA (0.25–1 μM) significantly decreased the amplitude of AMPA EPSCs in all cells tested (Fig. 2A). This inhibition was concentration dependent, with the highest concentration of KA (1 μM) producing an $86.0 \pm 4.4\%$ (mean \pm SE) inhibition of AMPA EPSCs, and reversed on wash out of KA.

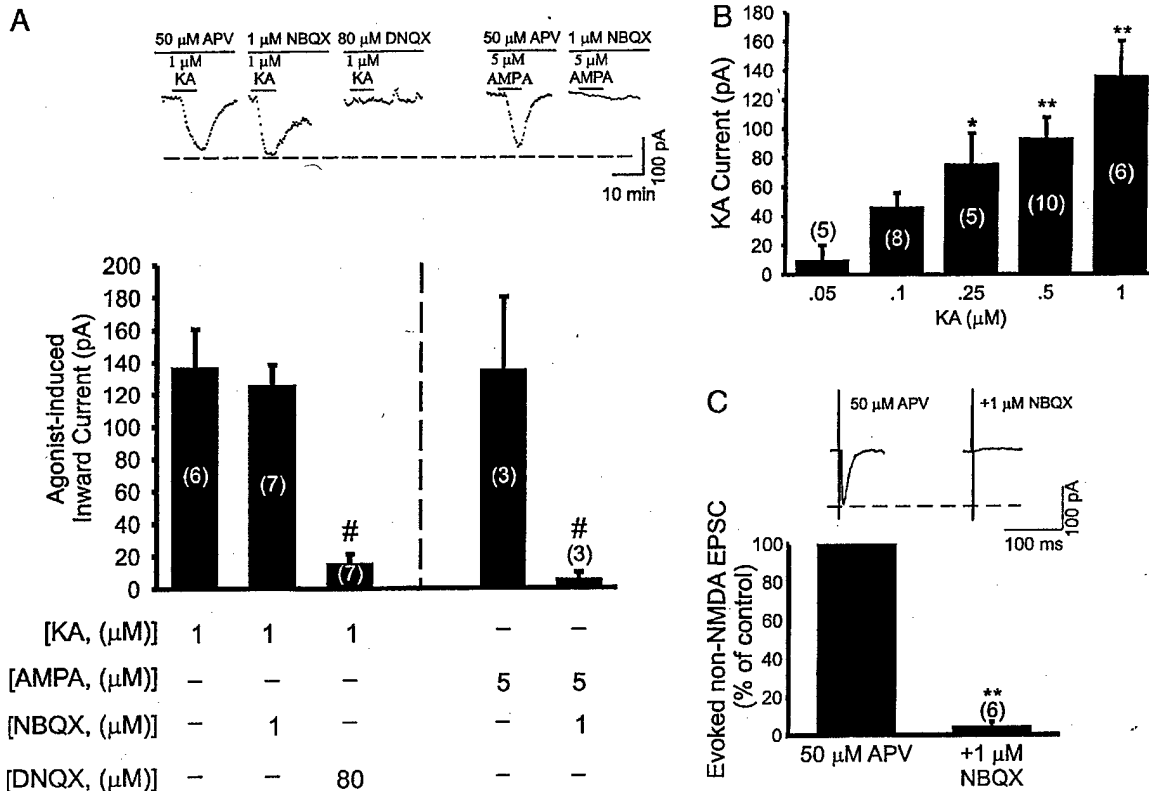


FIG. 1. Functional kainate receptors (KA-Rs) are present on nucleus accumbens (NAcc) core neurons. *A*: bar graph summarizing the average amplitude ($\text{pA} \pm \text{SE}$) of inward currents induced by the bath application of 1 μM KA or 5 μM α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) in NAcc neurons voltage clamped at -70 mV . The agonist-induced currents were evoked in the presence of 50 μM DL-(-)-2-amino-5-phosphonopentanoic acid (APV), 1 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) or 80 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX). The representative time courses presented above the graph show the inward current elicited by 1 μM KA in the presence of 50 μM APV, 1 μM NBQX, or 80 μM DNQX, and by 5 μM AMPA in the presence of 50 μM APV, and 1 μM NBQX. All recordings were done in the presence of 20 μM bicuculline (BIC). *B*: bar graph shows the average amplitude (in $\text{pA} \pm \text{SE}$) of inward currents evoked by bath application of KA (0.05–1 μM). *C*: non-*N*-methyl-D-aspartate (non-NMDA) excitatory postsynaptic currents (EPSCs) evoked by single pulse stimulation in the NAcc core are mediated solely by AMPA-Rs. Bar graph illustrates the effect of 1 μM NBQX on non-NMDA EPSCs, as percent of control $\pm \text{SE}$, in the presence of 50 μM APV and 20 μM BIC. Traces above the graph are averages of 7 non-NMDA EPSCs evoked in the absence and presence of NBQX (labeled 50 μM APV and 1 μM NBQX, respectively). Numbers in parentheses indicate the number of cells tested under each condition; significant difference from control: * $P < 0.05$ and ** $P < 0.001$; significant difference from agonist-induced current: # $P < 0.001$.

Since KA is an agonist at both KA-R and AMPA-Rs, we also characterized the effect of KA on glutamatergic synaptic transmission under conditions where AMPA-Rs were blocked by assessing the effect of KA on NMDA EPSCs. Pharmacologically isolated NMDA EPSCs were recorded in the presence of 20 μM BIC and an AMPA-R-selective concentration of NBQX (Fig. 2*B*). Similar to the effect of KA on AMPA EPSCs, a 7-min bath application of 1 μM KA resulted in a significant inhibition of NMDA EPSC amplitude ($60.0 \pm 9.5\%$ inhibition; Fig. 2*B*). Pretreating slices with the nonselective AMPA/KA receptor antagonist, DNQX, blocked the inhibitory effect of KA on NMDA EPSCs (Fig. 2*B*).

Kainate inhibits glutamatergic synaptic transmission via a presynaptic mechanism

To determine whether KA inhibited glutamatergic synaptic transmission in the NAcc via a pre- or postsynaptic mechanism, we performed two experiments. In the first experiment we examined the effect of KA on nonsynaptic glutamatergic

currents recorded from NAcc neurons. Currents were evoked every 60 s by local pressure application of 10 μM AMPA. These experiments were carried out in the continuous presence of 500 nM TTX to prevent the possible contribution of synaptic activity to the AMPA-evoked currents. A 7-min bath application of KA had a modest inhibitory effect on AMPA-evoked currents, reducing their amplitude by $26.2 \pm 6.0\%$ (Fig. 3). However, this inhibition was significantly less than the antagonism of AMPA EPSCs produced by the same concentration of KA recorded under similar experimental conditions.

In the second experiment, we examined whether KA-R activation altered the release probability for glutamate by determining the effect of KA on paired-pulse facilitation (PPF) of NMDA EPSCs. Two stimuli were paired with an interstimulus interval of 45 ms such that the second EPSC (*Peak 2*) was potentiated by the first EPSC (*Peak 1*). We then calculated the ratio of *Peak 2/Peak 1* in the absence and presence of 0.5 to 1 μM KA. In all cells tested, both *Peak 1* and *Peak 2* were inhibited by the application of KA; however, *Peak 1* was

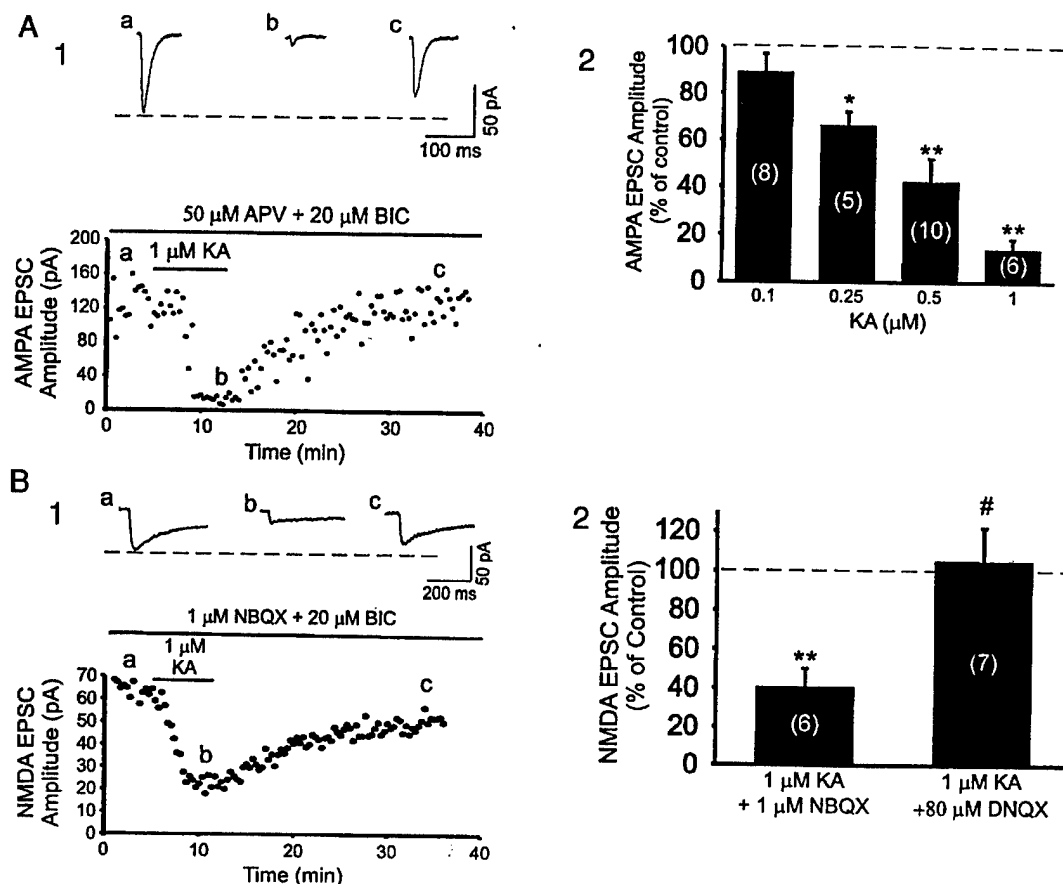


FIG. 2. KA-R activation inhibits glutamatergic synaptic transmission in the NAcc core. *A1*: time course of KA-R-mediated inhibition of AMPA EPSC amplitude (pA). Traces above the graph are averages of 7 EPSCs evoked at the times indicated by the corresponding letters in the graph. *A2*: summary bar graph showing the effect of bath application of KA (0.1–1 μ M) on AMPA EPSC amplitude as percent of control \pm SE. *B1*: time course of KA-R-mediated inhibition of NMDA EPSC amplitude (pA) recorded in the presence of 1 μ M NBQX and 20 μ M BIC. Traces above the graph are averages of 7 EPSCs evoked at the times indicated by the corresponding letters in the graph. *B2*: bar graph summarizes the effect of bath application of 1 μ M KA on NMDA EPSCs in the absence and presence of 80 μ M DNQX as percent of control \pm SE. Symbols are identical to those presented in Fig. 1, with the exception that # indicates significant difference from 1 μ M KA + 1 μ M NBQX, $P < 0.001$.

consistently inhibited to a greater extent. Thus bath application of KA significantly increased PPF (Fig. 4). Notably in three of eight cells tested, KA had a biphasic effect on *Peak 2*, potentiating *Peak 2* amplitude during the first few minutes and then inhibiting it during the latter phase of the KA application. This biphasic effect of KA was never observed with *Peak 1* in these paired-pulse experiments or with single EPSCs in any of the other experiments characterizing the presynaptic effects of KA.

Kainate inhibition of evoked EPSCs does not require indirect activation of mGluRs, GABA_B, or adenosine receptors

While the data presented so far are consistent with a presynaptic mechanism for KA inhibition of EPSCs in the NAcc, it does not necessarily follow that KA-Rs are localized to the presynaptic terminals of these synapses. A number of other

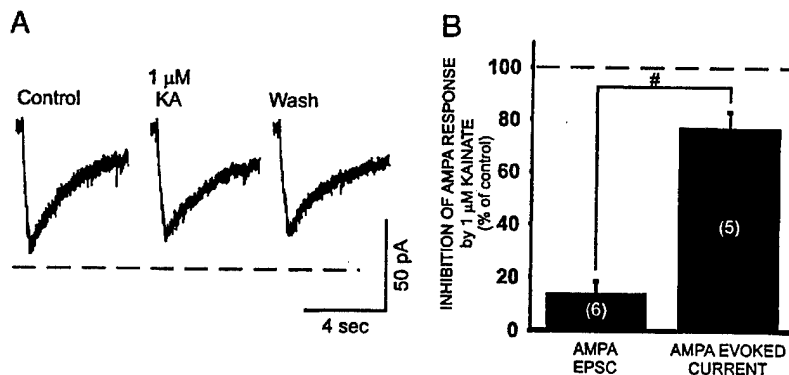


FIG. 3. KA-R activation does not inhibit nonsynaptic AMPA-R currents to the same extent as it inhibits AMPA-R-mediated EPSCs. *A*: representative traces demonstrating AMPA-R currents elicited by direct pressure application of 10 μ M AMPA in the absence and presence of 1 μ M KA. Traces are averages of 4 currents evoked by AMPA under baseline and KA conditions. *B*: summary bar graph comparing the inhibitory effect of 1 μ M KA on AMPA-R-mediated EPSCs (data from Fig. 2) and on currents evoked by local pressure application of 10 μ M AMPA. Symbols are identical to those presented in Fig. 1 with the exception that # indicates significant difference from AMPA EPSC, $P < 0.001$.

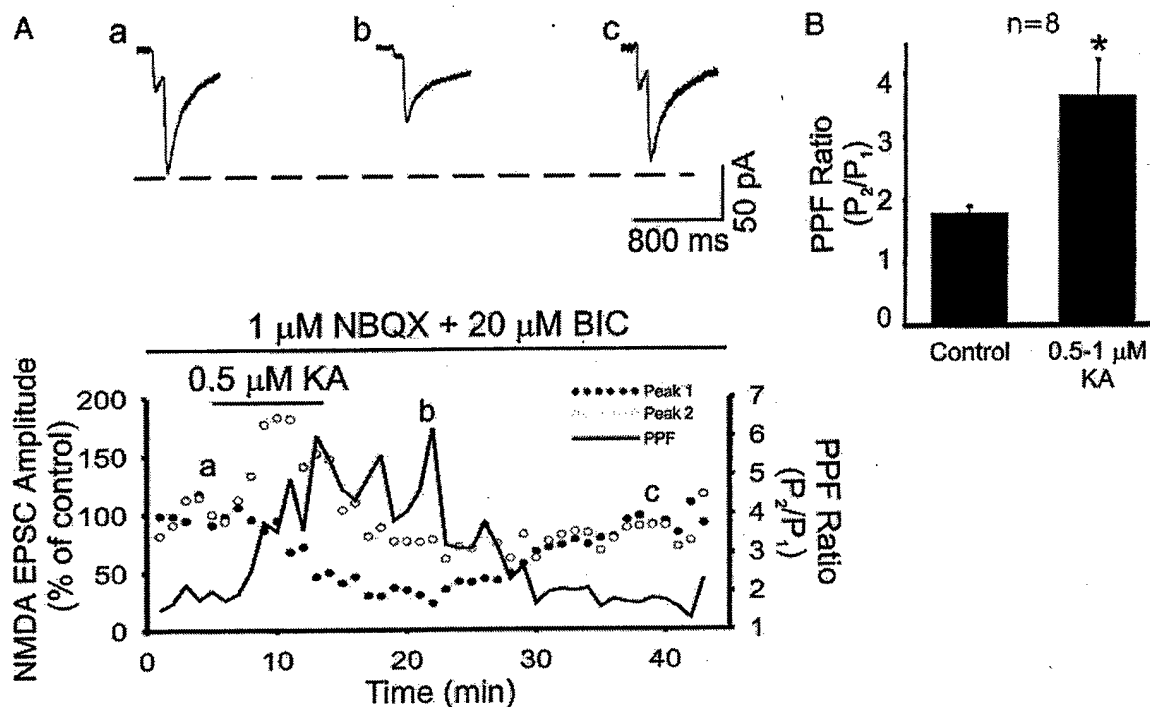


FIG. 4. KA-R activation increases paired-pulse facilitation (PPF) at glutamatergic synapses in the NAcc core. *A*: time course showing the increase in PPF of NMDA EPSCs in response to bath application of 0.5 μ M KA. Averaged traces above the graph represent paired NMDA EPSCs evoked at the times indicated by the corresponding letters in the graph. *B*: bar graph summarizing the PPF of NMDA EPSCs, expressed as the mean ratio of $P_2/P_1 \pm$ SE, in the absence and presence of KA. Symbols are identical to those presented in Fig. 1.

receptor systems have been hypothesized to indirectly mediate the presynaptic actions of KA, including mGluRs, GABA_B receptors, and adenosine receptors (Chergui et al. 2000; Frerking et al. 1999; Rodriguez-Moreno and Lerma 1998; Schmitz et al. 2001). For this reason, we sought to determine whether the KA-mediated inhibition of glutamatergic synaptic transmission, observed in the NAcc, was due to the secondary activation of these other receptor systems. To test this hypothesis, we pretreated ventral striatal slices with an mGluR antagonist cocktail (1 mM MCPG and 100 μ M CPPG), a GABA_B receptor antagonist (20 μ M SCH 50911), or a nonselective adenosine receptor antagonist (200 μ M theophylline) for 10 min prior to the bath application of KA. Pretreatment with MCPG/CPPG, SCH 50911, or theophylline had no significant effect on KA inhibition of glutamatergic synaptic transmission in the NAcc (Fig. 5).

DISCUSSION

This study sought to determine whether or not functional KA-Rs are present within the NAcc and to carry out an initial assessment of the physiological role of these receptors in regulating excitatory activity in this brain region. Our results provide the first demonstration that functional KA-Rs are expressed on neurons within the core of the NAcc. We found that these receptors can be activated by exogenous application of KA but they do not contribute to non-NMDA EPSCs evoked by individual stimuli. In addition, we present preliminary evidence suggesting that KA-Rs exert a potent presynaptic inhib-

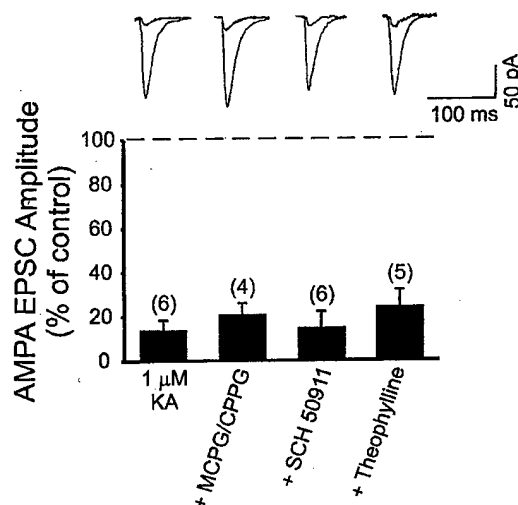


FIG. 5. KA-R-mediated inhibition of synaptic transmission at glutamatergic synapses in the NAcc core is not due to the secondary activation of presynaptic mGlu, GABA_B, or adenosine receptors. Bar graph summarizing the effect of 1 μ M KA on AMPA EPSC amplitude, expressed as percent of control \pm SE, in the absence and presence of the mGluR antagonists (RS)- α -methyl-4-carboxyphenylglycine (MCPG) and α -cyclopropyl-4-phosphonophenylglycine (CPPG; 1 mM and 100 μ M, respectively), the GABA_B antagonist SCH 50911 (20 μ M), or the adenosine receptor antagonist theophylline (200 μ M). Superimposed traces presented above the graph are averages of 7 AMPA EPSCs showing the effect of 1 μ M KA in the absence and presence of the antagonists. All traces were evoked in the presence of 50 μ M APV and 20 μ M BIC. Symbols are identical to those presented in Fig. 1.

itory effect on glutamatergic synaptic transmission in the NAcc.

In our first experiment, bath application of KA evoked an inward current in all NAcc core neurons recorded. Although KA is an agonist at both AMPA-Rs and KA-Rs, three lines of evidence suggest that these inward currents were mediated solely by the activation of KA-Rs. First, the concentrations of KA used in this study have previously been shown to selectively activate KA-Rs (Cossart et al. 1998). Second, a concentration of NBQX, that completely blocked AMPA-induced currents in the NAcc, and has been shown to be selective for AMPA-Rs in other native tissue preparations (Bureau et al. 1999), had no significant effect on KA currents in the NAcc. Finally, these KA-evoked currents were completely blocked by the mixed AMPA/KA receptor antagonist, DNQX. In the absence of commercially available selective KA-R antagonists, similar protocols have been used to demonstrate KA-R function in other brain regions (Frerking et al. 2001).

Despite the presence of agonist-induced KA currents in all NAcc cells tested, KA-Rs were not found to contribute to glutamatergic EPSCs evoked by individual stimuli in this study. This is consistent with studies in the CA1 region of the hippocampus and in the dorsal striatum, which demonstrated functional KA-Rs on postsynaptic cells but did not detect KA-R-mediated EPSCs (Bureau et al. 1999; Chergui et al. 2000). Although our data suggest that KA-Rs may be located extrasynaptically on NAcc neurons, the inability to elicit KA-R EPSCs in response to single stimuli does not rule out the possibility of synaptic KA-Rs in the NAcc. Evoking KA-R EPSCs in other brain regions has been shown to require the use of stimulus trains (Castillo et al. 1997; Li and Rogawski 1998; Vignes and Collingridge 1997). It will be important in future studies to determine whether higher frequency stimulation protocols can evoke KA-R mediated EPSCs in the NAcc.

In concordance with several studies conducted in the hippocampus (Contractor et al. 2000; Kamiya and Ozawa 1998, 2000; Schmitz et al. 2000), our data demonstrate that bath application of KA inhibits both AMPA-R- and NMDA-R-mediated EPSCs in the NAcc core. We also show that this inhibition requires KA-R activation by demonstrating that the effect was insensitive to AMPA-R inhibition but was completely blocked by mixed AMPA/KA-R antagonism. Overall, the effect of KA-R activation on AMPA and NMDA EPSCs was very similar. However, KA did have a slightly greater inhibitory effect on AMPA EPSCs. This difference may have been due to a relatively greater influence of postsynaptic shunting on AMPA EPSCs. Alternatively, the low concentration of NBQX used to isolate NMDA EPSCs may have partially inhibited the KA-Rs responsible for the inhibition of these responses.

We next sought to evaluate the mechanism(s) underlying KA-R-mediated inhibition of glutamatergic synaptic transmission in the NAcc. There is mounting evidence that the inhibition of synaptic transmission by KA in other brain regions is mediated, at least in part, by the activation of presynaptic KA-Rs (Contractor et al. 2000; Frerking et al. 2001; Kamiya and Ozawa 1998, 2000; Schmitz et al. 2000, 2001). While KA inhibition of glutamatergic EPSCs in this study showed a similar concentration dependence to the presynaptic inhibition characterized in other studies (see Schmitz et al. 2001), a

postsynaptic mechanism could also account for this effect. For example, it has been argued that KA-induced inward currents in the postsynaptic neuron could shunt I/EPSCs (as discussed in Contractor et al. 2000; Frerking et al. 1999). Although KA does induce inward currents in NAcc neurons, it is unlikely that a postsynaptic mechanism associated with shunting of incoming EPSCs is sufficient to account for the majority of the observed inhibition of glutamatergic EPSCs. Under our recording conditions, 1 μ M KA almost completely inhibited AMPA EPSCs evoked in NAcc neurons; however, this same concentration of KA had only a modest inhibitory effect on currents evoked by local pressure application of AMPA. These data suggest that the changes in the passive membrane properties of NAcc neurons induced by KA likely account for only a small component of the inhibition of EPSCs observed in the current study. This result is in agreement with studies in the hippocampus that show that postsynaptic shunting does not account for all of the KA-R-mediated inhibition of synaptic transmission at GABAergic synapses (Frerking et al. 1999). To further support the hypothesis that KA-R-mediated inhibition of glutamatergic synaptic transmission in NAcc neurons was due to a presynaptic action of KA, we examined the effect of KA on PPF. KA inhibition of EPSCs was associated with a significant increase in PPF in these cells. Increases in PPF have been shown to be a reliable indicator of a presynaptic decrease in the probability of neurotransmitter release (Manabe et al. 1993). Although this latter experiment does not exclude the contribution of a postsynaptic component to the inhibitory effect of KA on NAcc EPSCs, it further suggests that this inhibition results, at least in part, from a decrease in the release probability for glutamate.

The inhibitory effect of KA at glutamatergic synapses in the NAcc and other brain regions (Bortolotto et al. 1999; Frerking et al. 2001; Kamiya and Ozawa 1998, 2000; Vignes et al. 1998) may seem somewhat paradoxical in light of the known excitatory effects associated with KA-R activation (see Ben-Ari and Cossart 2000 for review). However, recent studies conducted in the hippocampus suggest that, under some experimental conditions, presynaptic KA-Rs at glutamatergic synapses may actually have a facilitatory effect on excitatory synaptic transmission (Bortolotto et al. 1999; Chittajallu et al. 1996; Contractor et al. 2000, 2001; Lauri et al. 2001b; Schmitz et al. 2001; see Huettner 2001 for review). For instance, reduced mossy fiber plasticity has been demonstrated in GluR6 knockout mice suggesting that KA-Rs act as presynaptic autoreceptors to facilitate synaptic transmission at these synapses (Contractor et al. 2001). In addition, exogenous activation of KA-Rs appears to have a biphasic effect on synaptic transmission at both the CA3-CA1 synapse (Chittajallu et al. 1996) and the mossy fiber synapse (Schmitz et al. 2001); where low concentrations of KA (<300 nM) facilitate, and high concentrations of KA (500–5,000 nM) inhibit neurotransmitter release (Schmitz et al. 2001). It has also been demonstrated that the activation of presynaptic KA-Rs contributes to the frequency facilitation observed at the mossy fiber synapse (Lauri et al. 2001b; Schmitz et al. 2001). Furthermore, Lauri et al. (2001a), suggest that facilitatory presynaptic KA-Rs are involved in long-term potentiation observed at mossy fiber synapses. In the current study, EPSCs evoked by single shock stimulation were always inhibited by KA-R activation, while

facilitation of NMDA EPSCs was occasionally observed in the PPF experiment, where *Peak 2* was transiently facilitated in a subset of cells. This observation might suggest that KA-R-mediated facilitation of EPSCs in NAcc neurons may be detected with lower concentrations of KA (<50 nM) or with different stimulation protocols. Thus the physiological role of presynaptic KA-Rs in the NAcc may be dependent on the magnitude and/or activation frequency of glutamatergic inputs.

A variety of models have been used to describe the possible mechanism(s) underlying the modulation of synaptic transmission by KA (for review see Ben-Ari and Cossart 2000; Frerking and Nicoll 2000). In the hippocampus, a number of studies have demonstrated direct presynaptic ionotropic (Schmitz et al. 2001) and metabotropic (Frerking et al. 2001; Rodriguez-Moreno and Lerma 1998) actions for KA-Rs. Studies at a number of different synapses in the hippocampus, as well as in other brain regions, suggest that the presynaptic effects of KA are mediated by the indirect activation of other receptor systems (Chergui et al. 2000; Frerking et al. 1999). For instance, KA could act at somatodendritic KA-Rs on local neurons to cause the release of neuromodulators, such as adenosine or GABA, which then act heterosynaptically on the terminals of glutamatergic synapses. Similar indirect mechanisms have been shown to mediate at least part of the apparent presynaptic effects of KA at inhibitory synapses in both the hippocampus (Frerking et al. 1999) and the dorsal striatum (Chergui et al. 2000). The mGluR system has also been hypothesized to underlie KA modulation of neurotransmitter release (Schmitz et al. 2001). The current study demonstrates that the inhibition of glutamatergic synaptic transmission by KA in the NAcc does not require the secondary activation of mGluRs, GABA_B receptors, or adenosine receptors. While these data support a direct presynaptic action for KA-Rs at glutamatergic synapses in the NAcc, it should be noted that the indirect activation of other receptor systems present in the ventral striatal slice could mediate the apparent presynaptic effect of KA in this brain region.

In the current study we observed no significant intercell variability in the response of EPSCs to KA-R activation. This result might suggest that there is a homogenous population of presynaptic KA-Rs present at all glutamatergic synapses in the NAcc. However, specific glutamatergic afferents in the coronal ventral striatal slice were not discretely activated in this study. Therefore, although many of the brain regions that provide glutamatergic innervation to the NAcc express KA-R subunits (Janssens and Lesage 2001; Meador-Woodruff et al. 2001), it is possible that these different excitatory inputs do not all possess presynaptic KA-Rs or contain presynaptic KA-Rs with different subunit compositions.

In summary, there is growing evidence that KA-Rs play an integral role in regulating synaptic activity in many brain regions (Ben-Ari and Cossart 2000; Frerking and Nicoll 2000). The current study suggests that functional KA-Rs are also present in the NAcc and that activation of these receptors may have both pre- and postsynaptic sequelae. Elucidation of the overall physiological role of KA-Rs in the NAcc will require further study. It will also be important to evaluate the possible involvement of these KA-Rs in the pathophysiology of disorders associated with perturbations in NAcc synaptic activity such as schizophrenia and drug and alcohol addiction.

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**Ethanol Antagonizes Kainate Receptor-Mediated
Inhibition of Evoked GABA_A IPSCs
in the Rat Hippocampal CA1 Region**

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List of Non-Standard Abbreviations: ACSF, artificial cerebrospinal fluid; APV, DL (-)-2-amino-5-phosphonovaleric acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EGTA, ethyleneglycol-bis-(β -aminoethyl ether) N,N,N,N'-tetraacetic acid; GABA, γ -aminobutyric acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPSC, inhibitory postsynaptic current.

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ABSTRACT

Many studies have demonstrated that ethanol reduces glutamatergic synaptic transmission primarily by inhibiting the NMDA subtype of glutamate receptor. In contrast, the other two subtypes of ionotropic glutamate receptor (AMPA and kainate) have generally been shown to be insensitive to intoxicating concentrations of ethanol. However, we have previously identified a population of kainate receptors that mediate slow EPSCs in the rat hippocampal CA3 pyramidal cell region that are potently inhibited by low concentrations of ethanol. In this study, we examined the effect of ethanol on kainate receptor-mediated inhibition of evoked GABA_A IPSCs in the rat hippocampal CA1 pyramidal cell region. Under our recording conditions, bath application of 1 μ M kainate significantly inhibited GABA_A IPSCs. This inhibition appeared to be mediated by the activation of somatodendritic kainate receptors on GABAergic interneurons and the subsequent activation of metabotropic GABA_B receptors, as the kainate inhibition was largely blocked by pretreating slices with a GABA_B receptor antagonist. Ethanol pretreatment significantly antagonized the inhibitory effect of kainate on GABA_A IPSCs, at concentrations as low as 20 mM. In contrast, ethanol did not block the direct inhibitory effect of a GABA_B receptor agonist on GABA_A IPSCs. The results of this study suggest that modest concentrations of ethanol may antagonize presynaptic, as well as postsynaptic, kainate receptor function in the rat hippocampus.

INTRODUCTION

Alcoholism represents an imposing medical and socio-economic concern for our society (Volpicelli, 2001). Surprisingly, little is known about the physiological factors that predispose an individual to this disease or the molecular mechanisms that mediate the intoxicating actions of ethanol. Recent studies have suggested that ethanol acts primarily by modulating the activity of a select group of neurotransmitter systems that mediate excitatory and inhibitory synaptic transmission (Faingold et al., 1998; Tsai and Coyle, 1998). It is thought that the summation of these multiple synaptic effects of ethanol underlies the complex behavioral sequelae associated with the intoxicating and reinforcing actions of this drug, and ultimately, the addiction process.

The majority of excitatory synaptic communication in the mammalian central nervous system (CNS) is mediated by the neurotransmitter glutamate. Glutamate activates three major classes of ionotropic receptors, named for the ligands α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), kainate, and N-methyl D-aspartate (NMDA) (Mayer and Westbrook, 1987). Given the central role that glutamate receptors play in numerous aspects of normal brain function, many studies have examined ethanol effects on glutamatergic synaptic transmission. To that end, there is now compelling evidence, from behavioral, neurochemical, and electrophysiological studies, that ethanol potently inhibits the activity of the NMDA subtype of glutamate receptor and that this inhibition contributes, in part, to some of the behavioral and cognitive effects of this drug (Tsai and Coyle, 1998; Woodward, 2000). In contrast, most studies have reported little or no effect of ethanol on glutamatergic responses mediated by non-NMDA (AMPA and kainate) receptors (e.g. Lovinger et al., 1990; Martin et al., 1991) (although see Nie et al., 1994; Martin et al., 1995; Valenzuela et al., 1998a).

Interestingly, in many of these earlier studies, it was not possible to distinguish between AMPA and kainate receptor-mediated responses. With the relatively recent development of

selective AMPA receptor antagonists (Paternain et al., 1995), it is now apparent that AMPA receptors are the primary mediators of fast excitation at most non-NMDA receptor-gated synapses. Thus, in many previous reports of ethanol insensitive non-NMDA receptors, responses were likely mediated predominantly by AMPA receptors.

The physiological role of the kainate subtype of glutamate receptor is only now beginning to emerge and little is known about the pharmacological properties of native kainate receptors. Although kainate receptors are widely expressed in the CNS, functional kainate receptor-gated synapses have only been identified in a limited number of brain regions (for reviews see Chittajallu et al., 1999; Frerking and Nicoll, 2000; Lerma et al., 2001). However, in addition to their somewhat limited postsynaptic role, functional presynaptic kainate receptors have been identified in a variety of brain areas. Activation of presynaptic kainate receptors has been shown to potently modulate neurotransmitter release in several brain regions, for example the hippocampus (Chittajallu et al., 1996; Cossart et al., 1998; Frerking et al., 1999) and the striatum (Chergui et al., 2000; Crowder and Weiner 2002).

We recently demonstrated that at least one population of kainate receptors in the rat hippocampus is sensitive to low concentrations of ethanol (Weiner et al., 1999). Ethanol, at concentrations as low as 20 mM, significantly inhibited kainate EPSCs recorded from rat hippocampal CA3 pyramidal neurons. In contrast, AMPA EPSCs in this brain region were insensitive to ethanol, even at the highest concentration tested (80 mM). These findings suggest that kainate receptors may represent a novel neuronal target of ethanol action in the mammalian CNS.

In the present study, we sought to determine if another kainate receptor-mediated response within the hippocampus might also be inhibited by intoxicating concentrations of

ethanol. We evaluated the effect of ethanol on kainate receptor-mediated inhibition of evoked GABA_A IPSCs (eIPSCs) in the rat hippocampal CA1 region. Recent evidence suggests that this effect is mediated by the activation of somatodendritic kainate receptors on presynaptic GABAergic interneurons (Cossart et al., 1998; Frerking et al., 1998) and that the subunit composition of these receptors may differ from that of the postsynaptic receptors underlying kainate EPSCs onto CA3 pyramidal neurons (Mulle et al., 2000). Our data suggest that ethanol, at concentrations similar to those that inhibit postsynaptic kainate receptors in the CA3 region, also inhibits kainate receptor-mediated inhibition of eIPSCs onto rat hippocampal CA1 pyramidal neurons. These results further support the hypothesis that native kainate receptors are significantly inhibited by relatively modest concentrations of ethanol and may potentially mediate some of the behavioral and cognitive effects of this drug.

METHODS

Hippocampal Slice Preparation

Transverse hippocampal slices (400 μ m) were prepared from 4-6 week old male, Sprague-Dawley rats as described previously (Weiner et al., 1997). Slices were incubated at ambient temperature (20-23°C) for ≥ 2 hours before recording in artificial cerebrospinal fluid (aCSF) containing (in mM): 126 NaCl, 3 KCl, 1.5 MgCl₂, 2.4 CaCl₂, 1.2 NaH₂PO₄, 11 glucose, and 26 NaHCO₃, saturated with 95% O₂-5% CO₂.

Electrophysiological Recordings

Slices were transferred to a recording chamber maintained at 20-23°C and superfused with aerated ACSF at 2 ml/min. Patch electrodes were prepared from filamented borosilicate glass capillary tubes (inner diameter: 0.86 mm) using a horizontal micropipette puller (Sutter P-

97; Sutter, Novato, CA). Electrodes were filled with a recording solution containing (in mM): KGlu, 130; KCl, 15; CaCl₂, 0.1; EGTA, 1.0; Mg-ATP, 2 (Sigma; St. Louis, MO); Tris-GTP, 0.2 (Sigma; St. Louis, MO); HEPES, 10; QX-314, 5; pH adjusted with KOH; 275-285 mOsm). Reagents used in the preparation of the recording solution were purchased from Fluka (Buchs, Switzerland) unless otherwise indicated. Whole-cell patch-clamp recordings were made from individual CA1 pyramidal neurons voltage clamped at -45 to -55 mV. Only cells with a stable access resistance of 5-20 M Ω were used in these experiments. Whole-cell currents were acquired using an Axoclamp 2B or Axopatch 200B amplifier, digitized (Digidata 1200B; Axon Instruments, Foster City, CA), and analyzed on- and off-line using an IBM compatible PC computer and pClamp 8.0 software (Axon Instruments, Foster City, CA).

Pharmacological Isolation of IPSCs

Evoked GABA_A receptor-mediated inhibitory postsynaptic currents were evoked every 20 seconds by electrical stimulation (0.2 msec duration) using a concentric bipolar stimulating electrode (FHC, Bowdoinham, ME) placed near the CA1 pyramidal cell body region ("proximal" stimulation; see (Weiner et al., 1997)). Unless otherwise indicated, GABA_A IPSCs were pharmacologically isolated using a cocktail of 50 μ M APV to block NMDA receptors and either 10 μ M LY 303070 (generous gift from Eli Lilly, Indianapolis, IN) or 1 μ M NBQX to block AMPA receptor function. 5 mM QX-314 (Alamone Labs, was included in the patch-pipette solution to block GABA_B IPSCs. Unless otherwise stated, all drugs used were purchased from Sigma (St. Louis, MO). A 4 M ethanol solution (AAPER, Shelbyville, KY; diluted in deionized water) was prepared immediately prior to each experiment from a 100% stock solution kept in a glass storage bottle. All drugs were applied directly to the ACSF via calibrated syringe pumps (Razel, Stanford, CT).

Statistics

All drug effects were quantified as the percent change in IPSC amplitude relative to the mean of control and washout values. Statistical analyses of drug effects were performed using the two-tailed Student's paired *t* test or a one-way analysis of variance followed by the Newman-Keuls post-hoc test with a minimal level of significance of $P < 0.05$.

RESULTS

Effect of Kainate on eIPSCs

We first examined the effects of exogenous kainate application on the amplitude of pharmacologically-isolated eIPSCs recorded from rat hippocampal CA1 pyramidal cells. Neurons were voltage-clamped at depolarized potentials (-45 to -55 mV) and eIPSCs were evoked every 20 seconds in the presence of the NMDA receptor antagonist APV (50 μ M) and the non-competitive AMPA receptor antagonist LY 303070 (10 μ M). We have previously shown that these concentrations of APV and LY 303070 completely block NMDA and AMPA EPSCs while having no significant effect on kainate receptor function in rat hippocampal neurons (Weiner et al., 1999). Synaptic currents evoked under these recording conditions were mediated solely by the activation of GABA_A receptors as they were completely antagonized by bath application of the selective GABA_A receptor antagonist bicuculline methiodide (data not shown). A 5-7 minute bath application of 1 μ M kainate significantly inhibited the amplitude of eIPSCs in all cells tested (to 34.3 ± 3.3 % of control, $N = 11$, $p < 0.01$) (Fig 1A, D). The onset of this inhibition was rapid and persisted for the duration of the kainate application. The effect was fully reversible upon washout with recovery taking between 20 to 45 minutes. Under these

recording conditions, the inhibition of eIPSCs by 1 μ M kainate was not accompanied by a significant change in holding current or input resistance.

It has been reported in murine studies that low concentrations of NBQX can be used to selectively antagonize AMPA receptor function (Bureau et al., 1999; Mulle et al., 2000). We therefore determined if a low concentration of NBQX was selective for AMPA over kainate receptors in the rat hippocampal CA1 region. Bath application of 1 μ M NBQX completely blocked AMPA EPSCs (by 97.8 ± 2.9 %; $N = 4$; data not shown) and notably, 1 μ M kainate had the same inhibitory effect on GABA_A IPSCs regardless of whether 1 μ M NBQX (38.5 ± 3.1 % of control, $N = 10$, $p < 0.01$) or 10 μ M LY303070 was used to antagonize AMPA receptor activity (Fig 1B, D).

We next sought to demonstrate that the inhibitory effect of kainate on eIPSCs required the activation of kainate receptors. Since selective kainate receptor antagonists are not commercially available, we used a protocol in which eIPSCs were first pharmacologically isolated using a blocker cocktail containing maximally effective concentrations of NMDA and AMPA receptor antagonists (either APV + LY 303070 or APV + NBQX). Slices were then perfused with a high concentration of the mixed AMPA/KA receptor antagonist DNQX and subsequently challenged with 1 μ M kainate. In the presence of the blocker cocktail, bath application of 80 μ M DNQX had no effect on the amplitude of eIPSCs (Fig 1C) suggesting that, under our recording conditions, there was no tonic kainate receptor-dependent regulation of GABAergic synaptic transmission. However, DNQX pretreatment completely blocked the inhibitory effect of exogenous kainate application on the amplitude of eIPSCs (94.7 ± 4.5 % of control, $N = 6$; $p > 0.05$)(Fig 1C, D).

Effect of Ethanol on Kainate Modulation of eIPSCs

We next examined the effect of ethanol on kainate inhibition of eIPSCs recorded in the presence of the AMPA and NMDA receptor antagonist blocker cocktail. Bath application of 80 mM ethanol significantly increased the amplitude and area of eIPSCs, as we have previously reported (Fig 2A) (Weiner et al., 1997). After a ten minute pretreatment in 80 mM ethanol, slices were then challenged with 1 μ M kainate in the continued presence of ethanol. Although kainate did significantly inhibit the amplitude of eIPSCs in the presence of 80 mM ethanol (to 73.6 ± 3.3 % of control, $N = 13$; $P < 0.05$), the magnitude of this inhibition was significantly less than that observed in the absence of ethanol ($P < 0.01$)(Fig 3). We next examined the concentration dependence of the ethanol antagonism of kainate-mediated inhibition of eIPSCs. Ethanol pretreatment produced a concentration dependent reduction of kainate-mediated inhibition of eIPSCs, with a significant effect being observed at 20 mM ethanol (to 55.3 ± 4.8 % of control, $N = 10$, $P < 0.05$), a concentration that had no effect on the amplitude or area of GABA_A IPSCs under these recording conditions (Figs 2B, 3).

Mechanism of Kainate Inhibition of eIPSCs

A number of mechanisms have been described to account for the inhibitory effect of kainate on eIPSCs in the rat hippocampus (for review, see Chittajallu et al., 1999; Frerking and Nicoll, 2000; Ben-Ari and Cossart, 2000; Lerma et al., 2001). A recent study demonstrated that the inhibitory effect of a relatively high concentration of kainate (10 μ M) on eIPSCs in the rat CA1 region could be blocked to a significant extent by pretreating slices with a GABA_B receptor antagonist (Frerking et al., 1999). The authors concluded that kainate activates somatodendritic kainate receptors on presynaptic GABAergic interneurons, resulting in a large increase in

spontaneous GABA release. This increased GABA release in turn activates presynaptic GABA_B receptors which are known to produce a pronounced decrease in evoked GABA release (Davies et al., 1990), therefore contributing to the kainate-mediated decrease in eIPSCs. To determine if a similar mechanism was responsible for the inhibitory effect of a lower concentration of KA, we tested the effect of 1 μ M kainate on eIPSCs in the presence of the GABA_B receptor antagonist SCH 50911. Under our recording conditions, bath application of 20 μ M SCH 50911 dramatically reduced the inhibitory effect of 1 μ M kainate on eIPSCs. In fact, kainate had no significant effect on eIPSCs in the presence of SCH 50911, reducing eIPSC amplitude to only 83.2 ± 6.7 % of control ($N = 11$; $P < 0.08$). This experiment suggests that the majority of the kainate inhibition of eIPSCs observed under our recording conditions is likely due to presynaptic kainate receptor-dependent release of GABA and the subsequent activation of presynaptic GABA_B receptors (Fig 4).

Effect of Ethanol on GABA_B Receptor-Mediated Inhibition of eIPSCs

The preceding experiment suggested that the inhibitory effect of 1 μ M kainate on GABA_A IPSCs was triggered by the activation of somatodendritic KA receptors on presynaptic GABAergic interneurons but also involved the secondary activation of presynaptic GABA_B receptors. We therefore sought to determine if ethanol was acting to inhibit the function of these interneuronal KA receptors or was, perhaps, acting downstream to antagonize presynaptic GABA_B receptor function. To differentiate between these two possible mechanisms, we directly assessed the effect of ethanol on presynaptic GABA_B receptor-mediated inhibition of GABA_A IPSCs. Under our recording conditions, bath application of 2.5 μ M baclofen, a selective GABA_B receptor agonist, significantly inhibited the amplitude of GABA_A IPSCs (to 42.2 ± 6.1 %, $N = 8$; $P < 0.001$)(Fig 5A). This inhibition was completely blocked by pretreating slices with the

GABA_B receptor antagonist, SCH 50911 (Figs 5A, C), suggesting that baclofen inhibition of GABA_A IPSCs was mediated by the activation of GABA_B receptors. We next tested the effect of 2.5 μ M baclofen in the presence of ethanol. As observed earlier, pretreating slices with 80 mM ethanol significantly potentiated GABA_A IPSCs. However, ethanol pretreatment did not block the inhibitory effect of baclofen on GABA_A IPSCs (Fig 7B). In fact, the inhibitory effect of 2.5 μ M baclofen was modestly enhanced in the presence of 80 mM ethanol (by $27.0 \pm 3.8\%$, $N = 9$; $P < 0.01$) (Fig 5C).

DISCUSSION

Previous work from our laboratory has demonstrated that relatively low concentrations of ethanol significantly inhibit postsynaptic kainate receptor function in rat hippocampal CA3 neurons (Weiner et al., 1999). The current study sought to evaluate the effect of ethanol on kainate receptor-mediated inhibition of eIPSCs in rat hippocampal CA1 pyramidal cells. Consistent with previous studies, we found that activation of kainate receptors by 1 μ M kainate significantly inhibited eIPSCs recorded from rat hippocampal CA1 pyramidal neurons. This inhibition involved the indirect activation of presynaptic GABA_B receptors, as pretreating slices with a GABA_B receptor antagonist blocked the inhibitory effect of kainate on eIPSCs. Pretreating slices with ethanol, at concentrations as low as 20 mM, significantly reduced kainate inhibition of eIPSCs. In contrast, ethanol did not antagonize the depressant effect of a GABA_B receptor agonist on eIPSCs. Taken together, these results demonstrate that, in addition to its inhibitory effect on postsynaptic kainate receptors in CA3 neurons, relatively modest concentrations of ethanol also significantly antagonize kainate receptor-mediated inhibition of GABAergic synaptic transmission in the CA1 region of the rat hippocampus.

Ethanol Inhibition of Interneuronal Kainate Receptor Function

In this study, bath application of ethanol significantly potentiated eIPSCs evoked by proximal stimulation, as we (Weiner et al., 1997) and others (Poelchen et al., 2000), have previously reported. This effect was primarily on the area of eIPSCs, and was significant at 40 and 80 mM ethanol. Bath application of 1 μ M kainate inhibited eIPSCs in the presence of ethanol, however the magnitude of this inhibition was significantly reduced at all but the lowest ethanol concentration tested (10 mM). Thus, ethanol antagonism of kainate inhibition of eIPSCs

appeared to be more potent than its direct potentiating effect on eIPSCs. Moreover, the potency of ethanol's depressant effect on kainate inhibition of eIPSCs was the same as that of ethanol antagonism of kainate EPSCs in CA3 pyramidal cells (Weiner et al., 1999). These data suggest that ethanol's overall facilitatory effect on proximal GABAergic synapses may be even more potent under physiological conditions in which presynaptic kainate receptors are active. Although we did not observe any regulatory effect of presynaptic kainate receptors on eIPSCs in the absence of exogenous kainate application in this study, synaptically released glutamate has been shown to modulate GABAergic synaptic transmission via activation of kainate receptors in other studies (Min et al., 2000; Jiang et al., 2001). In general, the synaptic activation of kainate receptors is most readily observed following intense or high frequency stimulation of glutamatergic afferents (see Frerking and Nicoll, 2000 for review). Therefore, when glutamatergic synaptic transmission is increased, for example during chronic ethanol withdrawal (Tsai and Coyle, 1998), ethanol inhibition of presynaptic kainate receptor function at GABAergic synapses may serve to further enhance the depressant effects of this drug on hippocampal function. Interestingly, there is some evidence that ethanol potentiation of eIPSCs is enhanced following chronic intermittent ethanol exposure (Kang et al., 1998).

As previously shown (Frerking et al., 1999), kainate receptor-dependent inhibition of eIPSCs at interneuron-CA1 pyramidal cell synapses in the current study appeared to be largely due to the secondary activation of presynaptic GABA_B receptors. It was therefore necessary to determine if ethanol was interacting with interneuronal kainate receptor function or rather with the presynaptic GABA_B receptors that are known to depress GABA release (Davies et al., 1990). We therefore tested whether ethanol had any effect on inhibition of eIPSCs mediated by the direct activation of presynaptic GABA_B receptors. Under our recording conditions, 2.5 μ M

baclofen inhibited eIPSCs to a similar extent as 1 μ M kainate. Pretreating slices with the highest concentration of ethanol tested in this study (80 mM) did not inhibit the effect of baclofen on eIPSCs. In fact, ethanol had a modest, but significant facilitatory effect on baclofen inhibition of eIPSCs and this novel interaction is currently under further investigation in our laboratory. Therefore, the inhibitory effect of ethanol on kainate inhibition of eIPSCs could not be attributed to an interaction between ethanol and presynaptic GABA_B receptors.

Postsynaptic shunting has also been shown to contribute significantly to the inhibition of eIPSCs by kainate receptor activation at interneuron-CA1 pyramidal cell synapses (Frerking et al., 1999). However, postsynaptic shunting did not appear to contribute to the inhibitory effect of kainate on eIPSCs in this study as bath application of 1 μ M kainate was not associated with any changes in input resistance or holding current. It should be noted that postsynaptic shunting of eIPSCs observed in the previous study was demonstrated with a kainate concentration ten times higher than that used in the present study. Lower concentrations of kainate have previously been reported to have only minimal effects on the passive membrane properties of CA1 pyramidal cells (Bureau et al., 1999).

Taken together these results further suggest that ethanol may interact directly with interneuronal kainate receptors, in a manner similar to its inhibitory effect on postsynaptic kainate receptors on CA3 pyramidal cells.

Ethanol Sensitivity of Non-NMDA Receptors

Our data suggest that hippocampal kainate receptors may be particularly sensitive to low concentrations of ethanol. These findings are somewhat surprising since few studies have demonstrated ethanol sensitive non-NMDA receptors in neuronal preparations (Martin et al.,

1991; and Nie et al., 1994). In the current study, as well as in another recent study from our laboratory (Weiner et al., 1999), ethanol was shown to potently inhibit pre- and postsynaptic kainate receptor function, but not AMPA receptor function, in the rat hippocampus. It might then be hypothesized that the ethanol sensitivity of non-NMDA receptors is dependent on the receptor subtype (i.e. kainate vs. AMPA) and/or on the subunit composition of these receptors. However, studies conducted with recombinant kainate receptors or native receptors in cultured cells suggest that this is unlikely. For example, Valenzuela and Cardoso (1999) demonstrated that the ethanol sensitivity of recombinant kainate receptors, unlike that of NMDA and GABA_A receptors (Masood et al., 1994; and Harris et al., 1997), does not vary with the particular subunits being expressed. Second, recombinant AMPA receptors expressed in either *Xenopus* oocytes (e.g. Dildy-Mayfield and Harris, 1992) or HEK 293 cells (Lovinger 1993), as well as AMPA receptors in primary culture (Wirkner et al., 2000) are potently inhibited by ethanol. Finally, studies conducted in cultured neurons have reported that ethanol inhibits both kainate and AMPA receptors, with little difference in the potency of these effects (Valenzuela et al., 1998a).

The factors responsible for the differential ethanol sensitivity of native non-NMDA receptors in tissue slices, native receptors in cultured cells, and recombinant receptors in expression systems are not known. One hypothesis is that receptors in these different environments might undergo differences in posttranslational modifications, such as phosphorylation, glycosylation, or protein-protein interactions, that might alter the ethanol sensitivity of these receptors. Posttranslational modifications have been shown to underlie changes in the ethanol sensitivity of NMDA receptors (see Chandler et al., 1998 for review). For example, phosphorylation reduces the ethanol sensitivity of these receptors during acute tolerance (Miyakawa et al., 1997). Although the ethanol sensitivity of kainate receptors appears

to be unaltered by phosphorylation (Valenzuela et al., 1998b), the effect of phosphorylation on the ethanol sensitivity of AMPA receptors has not been examined. Posttranslational modifications have been shown to account for other differences in the physiological and pharmacological properties of native and recombinant glutamate receptors (Standley and Baudry, 2000). For instance, modulation of glutamate receptor function by Concavalin A has been shown to require glycosylation (Everts et al., 1997). Differences in the posttranslational regulation of glutamate receptors in brain slices, cultured cells and expression systems, could contribute to previously observed differences in the ethanol sensitivity of these receptors. Clearly, further studies are needed to resolve the physiological mechanisms underlying the differential ethanol sensitivity of native and recombinant glutamate receptors.

Possible Behavioral Significance of Ethanol Inhibition of Interneuronal Kainate Receptors

Our data suggest that, at concentrations relevant to the pharmacological effects of ethanol, this drug may inhibit the activity of at least two populations of kainate receptors in the rat hippocampus. Assessing the behavioral significance of these observations at present is difficult as the physiological role of kainate receptors in this brain region is complex and not fully defined at the present time. For example, although kainate clearly inhibits eIPSCs, it likely does so via a profound excitation of presynaptic GABAergic interneurons and an associated increase in spontaneous GABA release. Moreover, recent data suggests that kainate may actually increase unitary eIPSCs under some conditions (Jiang et al., 2001). A further complication is that presynaptic kainate receptors also regulate glutamate release in the CA3 and CA1 regions and the ethanol sensitivity of these receptors remains to be determined. Nevertheless, studies with systemic administration or local infusion of kainate into the

hippocampus clearly indicate that the overall effect of kainate receptor activation on hippocampal physiology is profoundly excitatory in nature (Ben-Ari and Cossart, 2000). Therefore, it is likely that acute inhibitory effects of ethanol on hippocampal kainate receptor function will have a predominantly depressant effect on CNS activity, consistent with the known physiological sequelae associated with ethanol ingestion. Continued research into the physiological role of kainate receptors in the mammalian CNS will ultimately allow us to place the effects of ethanol on kainate receptor function into their proper context.

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Footnotes:

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FIGURE LEGENDS

Figure 1. Activation of kainate receptors inhibits eIPSCs in rat hippocampal CA1 pyramidal neurons. Summary time courses (6-11 cells) of the effect of a five-seven minute bath application of 1 μ M kainate (KA) on the amplitude of eIPSCs pharmacologically isolated with (A) 50 μ M APV and 10 μ M LY 303070 or (B) 50 μ M APV and 1 μ M NBQX (B). The summary time course in (C) illustrates that bath application of 1 μ M kainate has no effect on eIPSCs in the presence of a maximal concentration of the mixed AMPA/kainate receptor antagonist DNQX. Traces above each graph are averages of 5-8 eIPSCs recorded under the conditions indicated. (D) Bar graph summarizing the effect of bath application of 1 μ M kainate on the amplitude of eIPSCs recorded in the presence of the selective AMPA receptor antagonists LY 303070 and NBQX, and the mixed AMPA/kainate receptor antagonist DNQX. All recordings were carried out in the presence of 50 μ M APV. *, significant difference relative to control, $P < 0.05$. Numbers in brackets indicate the number of cells tested under each experimental condition.

Figure 2. Effect of ethanol on kainate inhibition of eIPSCs. Time course illustrating the effect of 80 mM (A) and 20 mM (B) ethanol on the inhibitory effect of 1 μ M kainate on eIPSCs recorded from a rat hippocampal CA1 pyramidal neuron. Traces above the graph are averages of 5-8 eIPSCs recorded at the times indicated by the letters.

Figure 3. Concentration dependence of ethanol antagonism of kainate inhibition of eIPSCs. *, $P < 0.05$, relative to the effect of 1 μ M kainate alone. Numbers in brackets indicate the number of cells recorded under each experimental condition.

Figure 4. Blockade of kainate inhibition of GABA_A IPSCs by the selective GABA_B receptor antagonist SCH 50911. A. Time course illustrating that 1 μ M kainate has no effect on the amplitude of eIPSCs in the presence of 20 μ M SCH 50911. Traces above the graph are averages of 5-6 eIPSCs recorded at the times indicated by the letters. B. Bar graph summarizing the effect 1 μ M kainate on the amplitude of eIPSCs in the absence and presence of 20 μ M SCH 50911. *, $P < 0.05$, relative to control. Numbers in brackets indicate the number of cells recorded under each experimental condition.

Figure 5. Ethanol does not antagonize kainate inhibition of GABA_A IPSCs via an indirect effect on presynaptic GABA_B receptors. (A) Time course of the effect of the GABA_B receptor agonist baclofen (BAC) on the amplitude of GABA_A IPSCs in the absence and presence of SCH 50911. Note that BAC inhibits the amplitude of GABA_A IPSCs and this effect is blocked by 20 μ M SCH 50911. (B) Time course illustrating the effect of 80 mM ethanol pretreatment on BAC inhibition of GABA_A IPSCs. Note that ethanol potentiates the amplitude and area of GABA_A IPSCs but does not occlude the inhibitory effect of BAC. (C) Bar graph summarizing the effect of bath application of 2.5 μ M BAC on the amplitude of GABA_A IPSCs alone, or following pretreatment with 80 mM ethanol or 20 μ M SCH 50911. *, $P < 0.05$, relative to control.

Figure 1

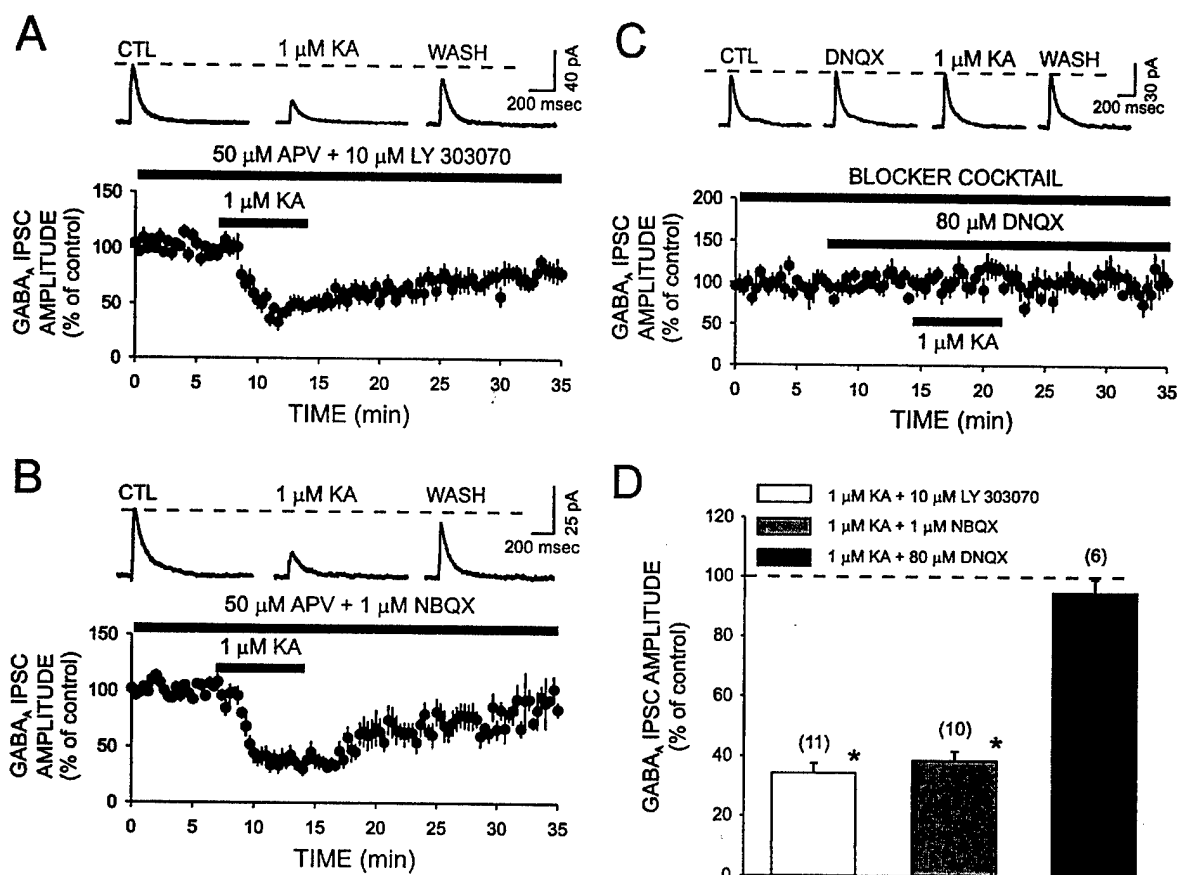


Figure 2

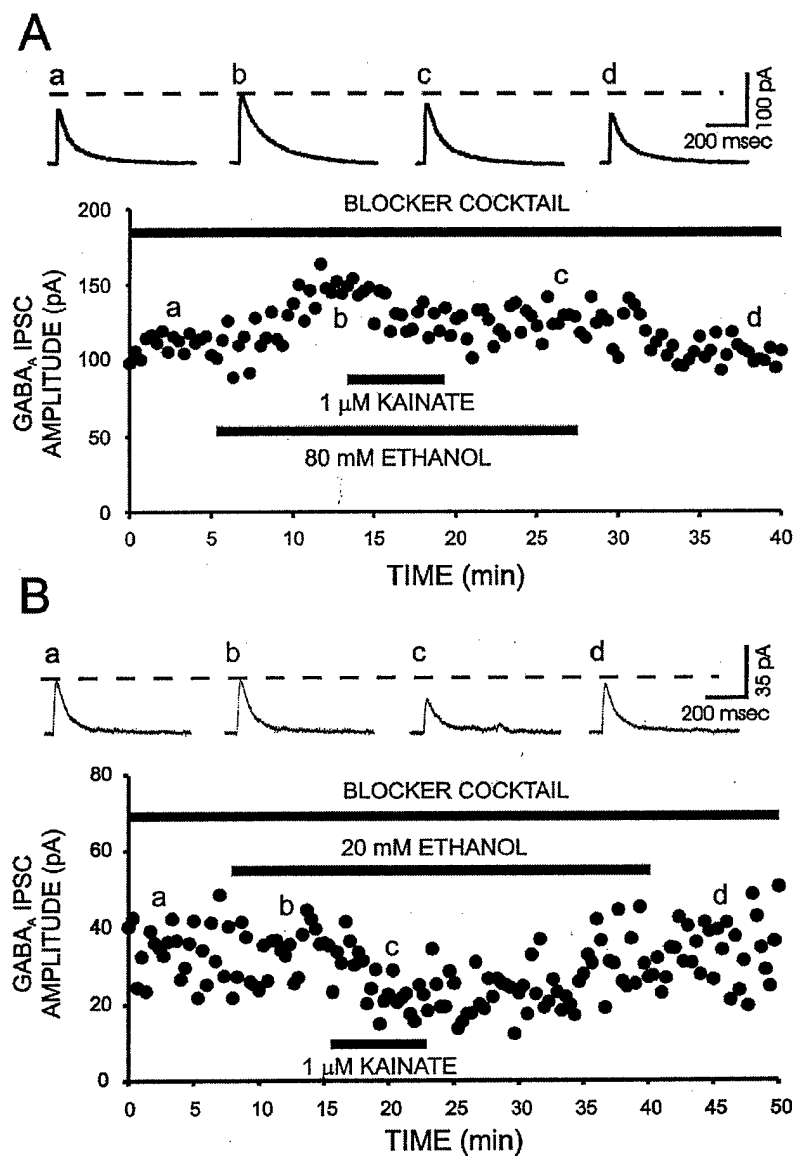


Figure 3

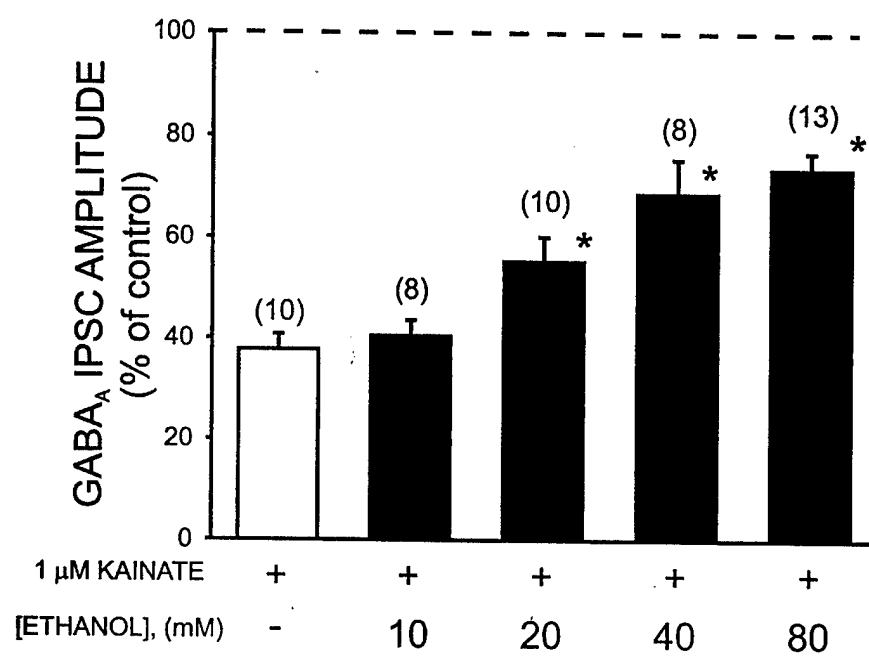


Figure 4

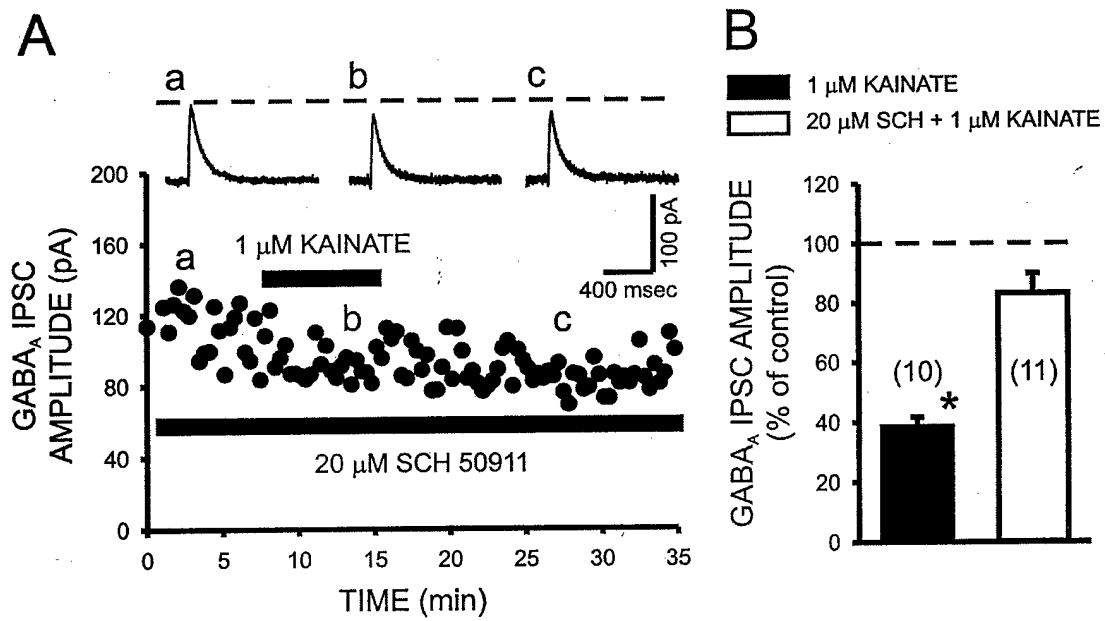
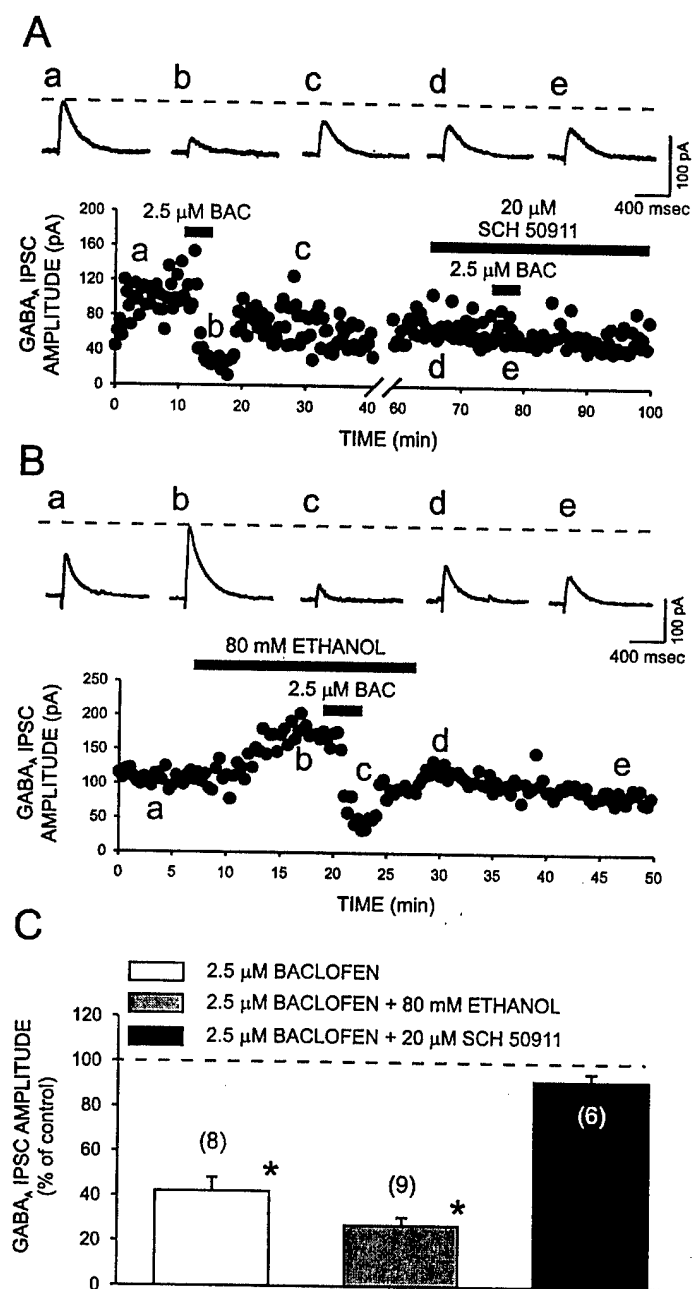


Figure 5



Kainate Receptor Activation Potentiates GABAergic Synaptic Transmission in the Nucleus Accumbens Core

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Running Title: Kainate Receptors at GABAergic Synapses in the NAcc

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Abstract

Inhibitory synaptic transmission plays an important role in regulating the activity of medium spiny neurons (MSNs) in the nucleus accumbens (NAcc). The kainate (KA) subtype of ionotropic glutamate receptor has recently been shown to potently modulate GABAergic synaptic transmission in several brain regions. Although KA receptor subunits are expressed in the NAcc, KA receptor modulation of GABAergic synaptic transmission in this brain region has not been previously examined. In the current study we sought to determine if KA receptor activation could alter inhibitory synaptic transmission in the NAcc as it does in other brain regions. Using the whole-cell patch clamp technique we demonstrate that KA receptor activation potentiates evoked GABAergic synaptic transmission and increases the frequency of spontaneous, but not miniature, GABA_A receptor-mediated IPSCs in the NAcc. In contrast, KA has no effect on currents evoked by exogenous application of GABA onto MSNs. Taken together these data suggest that activation of kainate receptors in the NAcc core potently facilitates action-potential dependent GABAergic synaptic transmission, likely via an excitation of presynaptic GABAergic interneurons.

Key Words: Nucleus Accumbens, Patch-clamp, IPSC, kainate, GABA, synaptic transmission, presynaptic.

The nucleus accumbens (NAcc) is the principal component of the ventral striatum and is comprised predominantly of γ -aminobutyric acid (GABA) containing projection neurons or medium spiny neurons (MSNs). It is well established that GABAergic synaptic transmission within the striatum plays a critical role in regulating the activity of these MSNs. Blockade of GABA_A receptors *in vivo* increases the firing rate of MSNs by at least 300% (Nisenbaum and Berger 1992), while activation of striatal GABA_A receptors can significantly decrease the responsiveness of MSNs to phasic changes in excitatory input (Kiyatkin and Rebec 1999). In addition to having physiological consequences, manipulation of striatal GABAergic synaptic transmission *in vivo* can produce substantial behavioral changes (Yoshida et al. 1991; Yamada et al. 1995). For example, altering inhibitory synaptic transmission within the NAcc has been shown to affect a variety of behaviors including locomotion (Wong et al. 1991; and Plaznik et al. 1992), feeding (Stratford and Kelley 1997), and ethanol self-administration (Rassnick et al. 1992; and June et al. 1998).

Several recent studies have highlighted an important role for kainate (KA) receptors, a subtype of ionotropic glutamate receptor, as presynaptic regulators of GABAergic synaptic transmission in a number of brain regions. These studies have demonstrated that exogenous KA receptor activation potently modulates GABA release in the hippocampus (Clarke et al. 1997; Rodriguez-Moreno et al. 1997; Cossart et al. 1998; Frerking et al. 1999; Min et al. 1999; Cuhna et al. 2000; Mulle et al. 2000; Rodriguez-Moreno et al. 2000; Ali et al. 2001; Cossart et al. 2001; Jiang et al. 2001; Semyanov and Kullman 2001), hypothalamus (Liu et al. 1999), neocortex (Ali et al. 2001), dorsal horn (Kerchner et al. 2001), as well as the dorsal striatum (Chergui et al. 2000). Further, in both the hippocampus and dorsal horn it has been shown that glutamate, synaptically released from nearby excitatory synapses, can activate KA receptors at GABAergic

synapses and mimic the effect of exogenous KA application on inhibitory synaptic transmission (Min et al. 1999; Cossart et al. 2001; Kerchner et al. 2001; and Semyanov and Kullman 2001). These findings suggest that KA receptors may play a significant role in the modulation of GABAergic synaptic transmission under physiologically relevant conditions.

A number of KA receptor subunits, including GluR6/7 and KA2, are highly expressed in the NAcc (Bischoff et al. 1997; and Kieval et al. 2001). Moreover, we (Crowder and Weiner 2002) and others (Casassus and Mulle 2002) have recently demonstrated that presynaptic KA receptors are present at glutamatergic synapses in the NAcc and that activation of these receptors potently inhibits evoked glutamate release. In the current study we tested the hypothesis that KA receptors would also regulate inhibitory synaptic transmission within the NAcc. Using the whole cell patch-clamp technique, we demonstrate that activation of KA receptors in the NAcc results in a modest potentiation of evoked inhibitory synaptic currents (eIPSCs) and a profound increase in the frequency of spontaneous IPSCs (sIPSCs) in all cells tested. Further, the KA receptor mediated potentiation of inhibitory synaptic transmission in the NAcc appears to be due to a presynaptic change and not an alteration in the responsiveness of the postsynaptic neuron to GABA. Finally, our data suggest that the KA receptor-mediated increase in inhibitory synaptic transmission in the NAcc is likely due to a facilitation of action-potential dependent GABA release and not a direct effect on neurotransmitter release at the presynaptic terminals.

Methods

Slice preparation: Male Sprague-Dawley rats (20-40 days old) were anesthetized with halothane and sacrificed by decapitation using a protocol approved by the ACUC of Wake Forest University School of Medicine. Coronal NAcc slices (400 μ m) were prepared using a vibrating tissue slicer (Leica VT1000S; Leica Instruments, Germany). Slices were then maintained at

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room temperature in oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 3.3 KCl, 2.4 MgCl₂, 2.5 CaCl₂, 1.2 KH₂PO₄, 10 d-glucose and 25.9 NaHCO₃, saturated with 95% O₂ - 5% CO₂. During recordings, slices were perfused with oxygenated aCSF at a flow rate of 2 ml/min. All drugs were applied through the aCSF in known concentrations via calibrated syringe pumps (Razel; Stamford, CT).

Patch-clamp recordings: Methods for whole cell recordings were similar to those reported previously (Weiner et al., 1999). Briefly, electrodes were prepared from filamented borosilicate glass capillary tubes (inner diameter: 0.86 mm) using a horizontal micropipette puller (Sutter P-97; Sutter, Novato, CA). Except during the recording of sIPSCs and miniature IPSCs, electrodes were filled with a recording solution containing (in mM): 130 K-gluconate, 10 KCl₂, 5 N-(2,6-dimethyl-phenylcarbamoylmethyl)-triethylammonium bromide (QX-314), 1 ethyleneglycol-bis-(β -aminoethyl ether) N,N,N,N'-tetraacetic acid (EGTA), 0.1 CaCl₂, 2 Mg-ATP, 0.2 tris-guanosine 5'-triphosphate, and 10 HEPES (free acid). During the recording of spontaneous activity, an equimolar concentration of CsCl₂ was substituted for K-gluconate in the internal solution. Whole cell patch-clamp recordings were made at room temperature from NAcc core neurons voltage-clamped between -20 and -40 mV. Recording electrodes were placed ventral to the anterior commissure within the core of the NAcc. Except during recordings in which the direct pressure application of GABA was used, synaptic currents were evoked every 20 seconds by electrical stimulation (0.2 msec duration) of tissue adjacent to the recording electrode using a concentric bipolar stimulating electrode (FHC, Bowdoinham, ME). Recordings were acquired with an Axoclamp 2B amplifier, digitized (Digidata 1200B; Axon Instruments, Foster City, CA), and analyzed on and off-line using an IBM compatible PC computer and pClamp 8.0 software (Axon Instruments, Foster City, CA). Spontaneous and miniature synaptic activity was

continuously acquired and stored in 3-minute epochs. Frequency and amplitude were analyzed off-line using an IBM compatible PC computer and MiniAnalysis software (Synaptosoft Inc).

Pharmacological Isolation: Drugs used in the pharmacological isolation of evoked currents included the NMDA receptor antagonist DL-(-)-2-amino-5-phosphonovaleric acid (APV), the GABA_A receptor antagonist bicuculline methbromide (BIC), the AMPA/KA receptor antagonists 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzof[*q*]quinoxaline-7-sulfonamide (NBQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (all from Sigma, St. Louis, MO). DNQX and NBQX were made up as stock solutions in dimethyl sulfoxide (DMSO) (final total concentration of DMSO was <0.05%). APV and BIC were made up as stock solutions in deionized water.

Pressure application of GABA: In some experiments, GABA (10 μ M) was applied directly to the soma of NAcc neurons using a Picospritzer III (General Valve, Fairfield, NJ). These experiments were carried out under visual guidance using an upright microscope equipped with differential interference contrast optics.

Statistics: KA effects on the amplitude of eIPSCs were defined as the percentage of control (pre-drug) values. KA effects on the frequency and amplitude of spontaneous and miniature IPSCs were also defined as the percentage of control (pre-drug) values. Student *t* tests were then used to analyze these data with significance set at $p < 0.05$.

Results

Effect of kainate on eIPSCs

In our first experiment, we sought to determine the effect of KA on evoked GABAergic synaptic transmission in the NAcc. Thus we tested the effect of 1 μ M KA on the amplitude of evoked GABA_A receptor-mediated inhibitory postsynaptic currents (eIPSCs) recorded from 18 MSNs in the NAcc. Bath application of 1 μ M KA potentiated the amplitude of eIPSCs in the

majority of cells tested (Figure 1A & B). Although, this effect was statistically significant, even with all cells included in the analysis, it was somewhat variable, with several cells showing little or no potentiation in the presence of KA. In the presence of a saturating concentration of the mixed AMPA/KA receptor antagonist, DNQX, 1 μ M KA had no effect on the amplitude of eIPSCs (Figure 1B).

Insert Figure 1
Here

Effect of kainate on spontaneous GABA_A IPSCs

We next sought to determine the effect of KA on spontaneous GABAergic synaptic transmission in the ventral striatum. We recorded spontaneous GABA_A receptor-mediated inward IPSCs (sIPSCs) from MSNs in the NAcc in the presence of 50 μ M APV, 1 μ M NBQX, and QX-314 to block NMDA, AMPA, and postsynaptic GABA_B receptors respectively. Under these conditions, the mean frequency of sIPSCs was 1.94 ± 0.24 Hz (mean \pm SEM; n= 26 cells). All spontaneous activity was blocked by the subsequent application of 20 μ M bicuculline, confirming that these events were mediated by the activation of GABA_A receptors (Figure 2A). Bath application of 1 μ M KA caused a significant increase in the frequency of sIPSCs in all cells tested (mean = 358 ± 61.2 %; $p < 0.01$, n= 7 cells) (Figure 2B & D). The increase in sIPSC frequency was maintained for the duration of the KA application and returned to baseline upon washout of KA. The application of KA had no effect on the amplitude of sIPSCs, with the amplitude of sIPSCs averaging 40.7 ± 3.9 pA and 44.25 ± 5.3 pA in the absence and presence of KA respectively (Figure 2C & D).

Insert Figure 2

Here

The KA-mediated increase in the frequency of spontaneous activity was concentration-dependent and was significant at concentrations as low as 0.1 μ M KA. Substituting 80 μ M DNQX for 1 μ M NBQX blocked the effect of 1 μ M KA on sIPSC frequency (Figure 3).

Insert Figure 3
Here

Effect of kainate on miniature GABA_A IPSCs

The results of the preceding experiments are consistent with a presynaptic action of KA receptor activation at GABAergic synapses in the NAcc. To further resolve the presynaptic locus of the KA receptors that mediated this effect, we recorded action potential-independent spontaneous GABAergic activity or miniature IPSCs (mIPSCs) in the presence of TTX to suppress action potential-dependent neurotransmitter release. The mean frequency of mIPSCs in the NAcc was 0.35 ± 0.03 Hz and the mean amplitude of these events was 20.5 ± 3.5 pA. In contrast to the effect of KA on action potential-dependent spontaneous activity, bath application of 1 μ M KA caused a modest, but significant, decrease in mIPSC frequency with no change in mIPSC amplitude (Figure 4A, B & D). Increasing the K⁺ concentration of the extracellular solution to 20 mM, a manipulation that should result in the depolarization of the presynaptic terminals, increased the frequency of mIPSCs (Figure 4C).

Insert Figure 4
Here

KA does not alter the responsiveness of postsynaptic NAcc neurons to GABA

To determine if activation of KA receptors on MSNs had any postsynaptic effects on GABA_A receptor function, we examined the effect of KA on currents evoked by exogenous application of GABA directly onto MSNs. Currents were evoked every 60 seconds by local pressure application of 10 μ M GABA. Bath application of 1 μ M KA had no significant effect on GABA-evoked currents (Figure 5).

Insert Figure 5
Here

Effect of external [Ca²⁺] on KA modulation of eIPSCs

To further study the mechanism by which KA potentiates inhibitory synaptic transmission in the NAcc, we altered the concentration of Ca²⁺ in the extracellular solution (aCSF) as has been done previously in the hippocampus and dorsal horn (Frerking et al. 1998; Rodriguez-Moreno 1998; Jiang et al. 2001; and Kerchner et al. 2001). In the first experiment, we raised the Ca²⁺ concentration of the aCSF from 2 to 7 mM (high Ca²⁺). This manipulation is thought to reduce neuronal excitability by closing voltage-dependent channels (increasing the resting membrane resistance), and raising the threshold for electrical excitation (Hille 1992). Consistent with a decrease in neuronal excitability, high Ca²⁺ reduced the amplitude of eIPSCs recorded from MSNs in the NAcc. Under these high Ca²⁺ conditions, subsequent bath application of 1 μ M KA caused a significant increase in eIPSC amplitude in all cells tested (Figure 6A & C). In the second experiment, the Ca²⁺ concentration of the aCSF was lowered from 2 to 0.5 mM (low

Ca^{2+}). This manipulation is thought to increase neuronal excitability (Hille 1992). Under these conditions, addition of 1 μM KA had no effect on eIPSC amplitude in any cells tested (Figure 6B & C).

Insert Figure 6
Here

Discussion

The role of KA receptors in the central nervous system has only recently begun to be examined. Recent studies have highlighted an important presynaptic role for KA receptors in regulating neurotransmitter release at both excitatory and inhibitory synapses (see Frerking and Nicoll 2001; and Kullmann 2001 for review). Within the NAcc, activation of KA receptors has been shown to potently inhibit excitatory synaptic transmission (Casassus and Mulle 2002; and Crowder and Weiner 2002). The current study sought to determine if KA receptors would also modulate inhibitory input to MSNs in the NAcc. Our results suggest that activation of KA receptors at inhibitory GABAergic synapses in the NAcc results in a significant potentiation of evoked and spontaneous GABAergic synaptic transmission. In addition, we have demonstrated that the enhancement of GABAergic inhibition appears to be mediated by a presynaptic mechanism that is dependent on action potential-dependent neurotransmitter release.

As mentioned above, many studies have examined the effect of KA receptor activation on GABAergic synaptic transmission in several different brain regions (Cossart et al. 1998; Frerking et al. 1999; Liu et al. 1999; Chergui et al. 2000; Cuhna et al. 2000; Mulle et al. 2000; Rodriguez-Moreno et al. 2000; Cossart et al. 2001; Jiang et al. 2001; Kerchner et al. 2001; and Semyanov and Kullman 2001). In the majority of these studies, bath application of KA has been shown to result in a significant increase in spontaneous GABA release that is usually

accompanied by a significant decrease in the amplitude of evoked GABA_A IPSCs. Such effects have been shown in the hippocampus (see Mody 1998 for review), dorsal horn (Kerchner 2001), and dorsal striatum (Chergui et al. 2000). Although the mechanisms underlying these effects have not been fully resolved, the increase in the frequency of spontaneous activity is generally attributed to a direct ionotropic action of KA receptors, depolarizing either the soma, dendrites or axon of presynaptic cells, thus increasing firing rate (somatodendritic/axonal receptors) (Cossart et al. 1998; Frerking et al. 1998; Chergui et al. 2000; Semyanov and Kullman 2001) or depolarizing the presynaptic terminals thereby increasing neurotransmitter release (terminal receptors) (Liu et al. 1999; Mulle et al. 2000; Cossart et al. 2001; and Kerchner et al. 2001). In contrast, the mechanism underlying the associated inhibition of eIPSCs is less clear. Initial reports suggested that KA receptor activation increases interneuronal activity leading to a use-dependent depression of the eIPSCs (Frerking et al. 1998). Subsequent reports have demonstrated that the inhibition of eIPSCs is, at least in part, due to indirect effects of KA receptor activation, mediated by the secondary activation of other presynaptic receptor systems, including GABA_B or adenosine receptors, and postsynaptic shunting of eIPSCs (Frerking et al. 1999; Chergui et al. 2000; see Frerking and Nicoll 2000 for review). Moreover, there is some evidence that KA receptors inhibit eIPSCs via the activation of second messenger cascades directly coupled to the receptors at presynaptic terminals (Cuhna et al. 2000; Rodriguez-Moreno et al. 2000; and Lerma et al. 2001).

In the current study we found that bath application of a low concentration of KA produced a significant potentiation of eIPSC amplitude in the NAcc. Although this effect was significant, it was variable, with some cells showing no effect of KA on eIPSC amplitude. In contrast to the somewhat variable effects of KA on eIPSCs, KA receptor activation resulted in a

significant increase in sIPSC frequency in all cells tested. This increase in sIPSC frequency was not accompanied by any change in sIPSC amplitude suggesting a presynaptic locus of KA action. In addition, we found that the KA-mediated increases in eIPSC amplitude and sIPSC frequency, observed in the presence of an AMPA receptor selective concentration of NBQX (see Crowder and Weiner 2002), were blocked in the presence of a saturating concentration of a non-selective AMPA/KA receptor antagonist. Thus, the KA-induced facilitation of GABAergic synaptic transmission in the NAcc required the selective activation of KA receptors.

The effect of KA on sIPSCs observed in the NAcc is consistent with studies conducted at inhibitory synapses in the hippocampus (e.g. Semyanov and Kullmann 2001). This KA-induced increase in spontaneous activity may have resulted from a direct ionotropic effect via presynaptic KA receptors located at the terminals (Liu et al. 1999) or via somatodendritic/axonal KA receptors (Cossart et al. 1998; and Frerking et al. 1999; Semyanov and Kullmann 2001). We tested the former hypothesis by examining the effect of KA on action potential independent sIPSCs (mIPSCs). In contrast to the effect of KA on sIPSCs, we found that KA did not increase the frequency of mIPSCs. In fact we observed a modest decrease in mIPSC frequency. Thus, the large KA-mediated increase in sIPSC frequency observed in our earlier experiments could not be due to the depolarization of the presynaptic terminals. This conclusion is further supported by the observation that mIPSC frequency could be increased under our recording conditions by depolarizing the terminals via an elevation of the extracellular K^+ concentration. Taken together these data indicate that the KA receptor mediated-increase in GABAergic synaptic transmission was dependent upon action potential dependent neurotransmitter release, likely via the activation of somatodendritic or axonal KA receptors.

Given the robust potentiating effect of KA on sIPSCs observed in all cells tested, it seems somewhat surprising that KA resulted in a modest, but significant decrease in action potential-independent mIPSC frequency in the current study. Although most studies in other brain regions have consistently reported a KA-mediated facilitation of sIPSC frequency, the effects of KA on mIPSCs have been more variable. For instance, Frerking et al. (1998, 1999) reported no effect of KA on mIPSC frequency or amplitude at interneuron to CA1 synapses in the hippocampus. In contrast, Rodriguez-Moreno et al. (1998) reported a significant inhibition of mIPSC frequency at the same hippocampal GABAergic synapses. These authors suggested that perhaps a second, distinct population of KA receptors that act to decrease the release of GABA might be localized to the presynaptic terminals (Rodriguez-Moreno 2000). Further investigation will be required to determine the mechanisms underlying the effect of KA on mIPSCs in the NAcc. Nevertheless, this experiment clearly indicates that the large KA-mediated increase in sIPSC frequency observed in this study cannot be attributed to KA receptors localized to the presynaptic terminals of these synapses.

Under our recording conditions, bath application of KA resulted in a modest, but significant, increase in the amplitude of eIPSCs recorded from MSNs in the NAcc. In contrast, bath application of KA had no effect on the amplitude of s- or mIPSCs, nor did it alter the amplitude of currents evoked by exogenous application of GABA. These data suggest that KA increased eIPSC amplitude via a presynaptic mechanism, likely related to the profound increase in sIPSC frequency. Since KA-induced increases in sIPSC frequency are generally associated with inhibition of eIPSCs in other brain regions, the data presented here suggest that there may be fundamental differences between the properties of GABAergic synapses in the NAcc

compared with those in other brain regions like the hippocampus, hypothalamus, dorsal horn and dorsal striatum.

We postulated two possible hypotheses to explain why KA increases the amplitude of eIPSCs in the NAcc but inhibits eIPSC amplitude in most other brain regions: 1) the KA-induced inhibition of eIPSCs in other brain regions is mediated by the secondary activation of other receptor systems not present in the NAcc; 2) the effect of KA on eIPSC amplitude is dependent on the basal level of presynaptic neuronal excitability and this parameter is lower in the NAcc than in other brain regions like the hippocampus

As mentioned earlier, KA inhibition of eIPSCs is significantly reduced by pretreating slices with a GABA_B receptor antagonist in the hippocampus and dorsal horn (Frerking et al. 1999; and Kerchner et al. 2001) and by pretreatment with an adenosine receptor blocker in the dorsal striatum (Chergui et al. 2000). Therefore it is possible that these indirect mechanisms may not be activated by kainate in the NAcc thereby revealing a potentiation of eIPSCs. However, there are several reasons why this hypothesis is unlikely. First, even when these indirect pathways are blocked in other brain regions, KA does not produce a potentiation of eIPSC amplitude. Second, both GABA_B and adenosine receptor modulation of GABAergic synaptic transmission have been demonstrated in the NAcc (Uchimura and North, 1990; Tao et al. 1996; Harvey and Lacey 1997; and Steffensen et al. 2000). However, pretreatment with antagonists for both of these receptor systems (SCH 50911; and theophylline; GABA_B and adenosine receptor antagonists, respectively) had no effect on the KA receptor-mediated potentiation of eIPSCs observed in the current study (data not shown). Therefore, while it is clear that the secondary activation of other receptor systems contributes to the KA-induced inhibition

of eIPSCs in some brain regions, this explanation cannot fully account for the differential effects of KA on eIPSC amplitude observed between the NAcc and these other brain regions.

Alternatively, we hypothesized that the differential effects of KA on eIPSC amplitude may be related to basal excitability. For example, while, Frerking et al. (1999) demonstrated that the inhibition of eIPSCs in the hippocampus was also partially accounted for by decreases in postsynaptic responsiveness to GABA, decreases in eIPSC amplitude due to alterations in the excitability of the presynaptic GABAergic neurons could not be ruled out. The basal frequency of sIPSCs in the NAcc is considerably lower than that observed in the hippocampus (Hoffman and Lupica, 2001). Thus the differential effect of KA on eIPSC amplitude might be explained if KA only enhances eIPSCs when presynaptic neuronal excitability is relatively low. It is well established that manipulating the divalent ion concentrations of the extracellular solution can alter neuronal excitability such that increasing extracellular Ca^{2+} decreases neuronal excitability whereas decreasing extracellular Ca^{2+} increases neuronal excitability (Hille 1992). We therefore tested the effect of KA on eIPSCs in high and low extracellular Ca^{2+} . As expected, in high Ca^{2+} , bath application of KA significantly increased eIPSC amplitude in all cells tested. In contrast, in low Ca^{2+} , KA had no effect on eIPSCs. This experiment suggests that KA receptor activation increased evoked GABAergic transmission in the NAcc only under conditions where neuronal excitability was low.

Interestingly, Kerchner et al. (2001) recently demonstrated that KA potentiation of eIPSC amplitude could also be revealed in the dorsal horn when high Ca^{2+} was used to reduce neuronal excitability. Similar manipulations of divalent ion concentrations have been conducted in the hippocampus (Rodriguez-Moreno 1998; and Frerking et al. 1998) where basal neuronal activity is relatively high. Reducing neuronal excitability of hippocampal cells prevents the paradoxical

inhibition of eIPSCs by KA receptor activation. Taken together these studies suggest that the effect of KA receptor activation on eIPSCs may, at least in part, be dependent on neuronal excitability.

This hypothesis could be ruled out if KA depressed eIPSC amplitude in neurons with basal sIPSC frequencies similar to those demonstrated in the NAcc. While, KA was shown to inhibit eIPSC amplitude in the dorsal striatum (Chergui et al. 2000) where the basal sIPSC frequency is similar to that in the NAcc, the KA-induced increase in sIPSC frequency in the dorsal striatum was almost 2-fold higher than the increase reported in the current study.

Since KA increased eIPSCs in the presence of a physiological concentration of Ca^{2+} in our initial experiments, these data are consistent with the hypothesis that the excitability of GABAergic interneurons in the NAcc may be relatively low under our standard recording conditions. Of course this doesn't rule out the possibility that the potentiating effects of KA on evoked GABAergic synaptic transmission in the NAcc are mediated by an as yet unidentified indirect mechanism.

Significance

Despite anatomical evidence for extensive axon collateral networks between the primary projection neurons in the NAcc (Wilson and Groves 1980), electrophysiological studies suggest that functional GABAergic input to MSNs predominantly originates from local interneurons (Jaeger et al., 1994). It has been hypothesized that these GABAergic interneurons in NAcc are a component of a neural circuit whereby cortical and hippocampal information may be passed to MSNs in the form of inhibition and thus form the anatomical substrate for feed-forward inhibition (Bennett and Bolam 1994; and Pennartz and Kitai 1991). Given the recent demonstration that KA receptor activation decreases glutamatergic synaptic transmission

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(Casassus and Mulle 2002; and Crowder and Weiner 2002) and the current demonstration that KA receptor activation potentiates GABAergic synaptic transmission, activation of presynaptic KA receptors in NAcc would result in an inhibition of MSNs activity and thus an inhibition of NAcc output. Whether KA receptors play a significant role in behaviors that may be associated with reductions in NAcc output, i.e. alcohol and reward (Carlezon and Wise 1990; Criado et al. 1995), locomotion (Yang and Mogenson 1989), and/or schizophrenia (Swerdlow et al. 1990), requires further investigation.

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Figure 1: KA alters evoked GABAergic synaptic transmission in the NAcc core. *A*:

Representative time course demonstrating the potentiation of eIPSC amplitude produced by bath application of 1 μ M KA in the presence 1 μ M NBQX and 50 μ M APV. Traces above the graph are averages of 7 traces taken at the times indicated by the corresponding letters in the time course. *B*: Bar graph illustrating the average effect of 1 μ M KA, expressed as percent of control \pm SEM, on the amplitude of eIPSCs in all cells tested, in the presence of either NBQX or DNQX. Numbers in brackets indicate the number of cells under each condition; * indicates significant difference from control $p < 0.05$.

Figure 2: KA increases spontaneous GABA_A IPSCs frequency in the NAcc core. *A*:

Representative traces demonstrating the effect of 1 μ M KA on sIPSCs recorded in the presence of 1 μ M NBQX, 50 μ M APV and QX-314 from MSNs in the NAcc. The effect of KA reverses upon washout of KA and all spontaneous activity is blocked by the subsequent addition of 20 μ M bicuculline. *B*: Cumulative histogram of sIPSC interevent interval in control conditions (solid line) and in the presence of 1 μ M KA (dotted line) is shown for the representative recording. *C*: Cumulative histogram of sIPSC amplitude in control conditions (solid line) and in the presence of 1 μ M KA (dotted line) is shown for the representative recording. *D*: Bar graph summarizing the effect of bath application of 1 μ M KA on sIPSC frequency and amplitude shown as percent of control \pm SEM. Numbers in parentheses indicate the number of cells tested under each condition; ** indicates significant difference from control $p < 0.01$; * indicates significant difference from control $p < 0.05$.

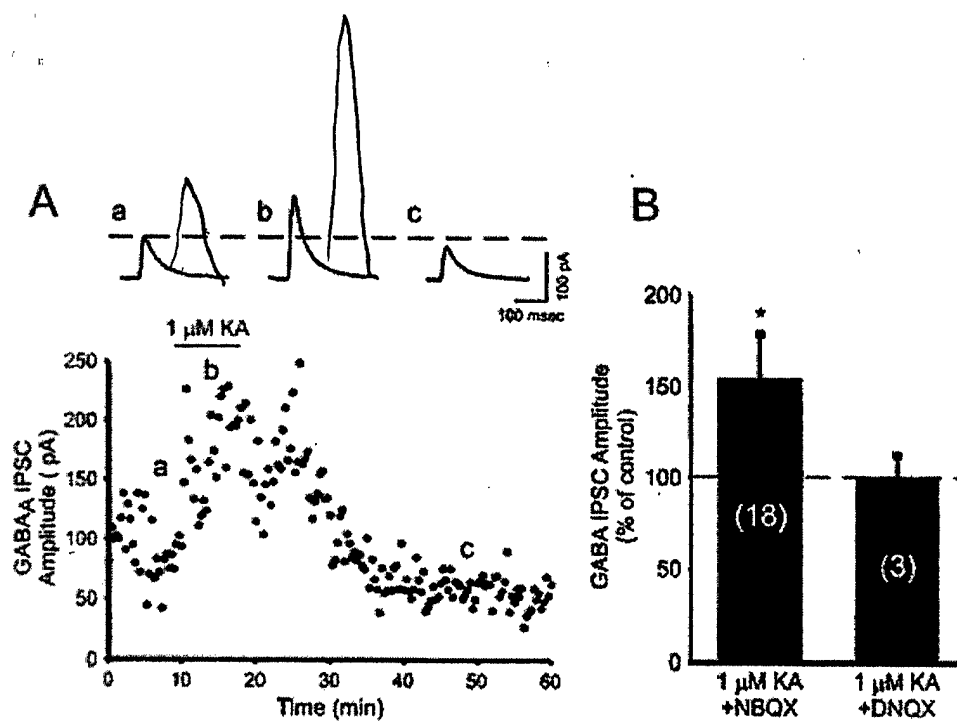
Figure 3: KA-mediated increase in sIPSC frequency is concentration dependent and requires the specific activation of KA receptors. Bar graph summarizing the effect of (0.01-1 μ M KA) on sIPSC frequency in the presence of 1 μ M NBQX, and the effect of 1 μ M KA on sIPSC frequency in the presence of 80 μ M DNQX, shown as percent of control. Representative traces above the graph demonstrate the effect of 1 μ M KA on sIPSCs in the presence of 80 μ M DNQX. Numbers in parentheses indicate the number of cells tested under each condition. * * indicates a significant difference from control, $p < 0.01$; * indicates a significant difference from control, $p < 0.05$; # indicates a significant difference from 1 μ M KA (in the presence of NBQX) ($p < 0.01$).

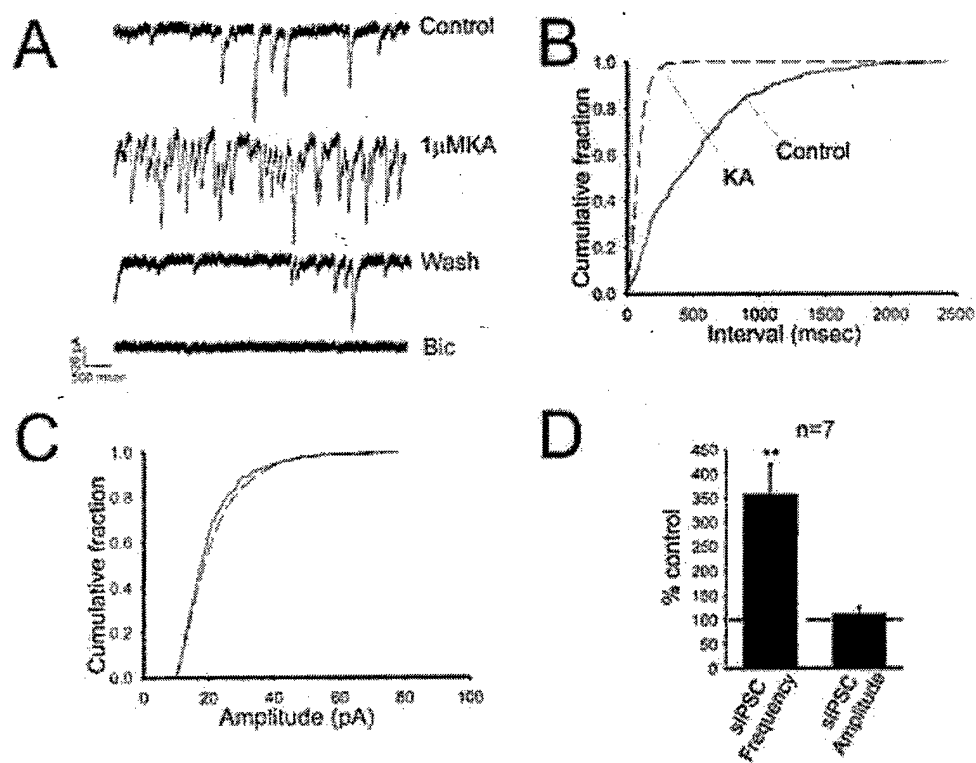
Figure 4: KA does not increase mIPSC frequency in the NAcc. *A*: Representative traces of mIPSCs recorded in NAcc neurons in the absence and presence of 1 μ M KA *B*: Bar graph summarizing the effect of 1 μ M KA on mIPSC frequency and amplitude in the NAcc. * indicates significant difference from control, $p < 0.05$.

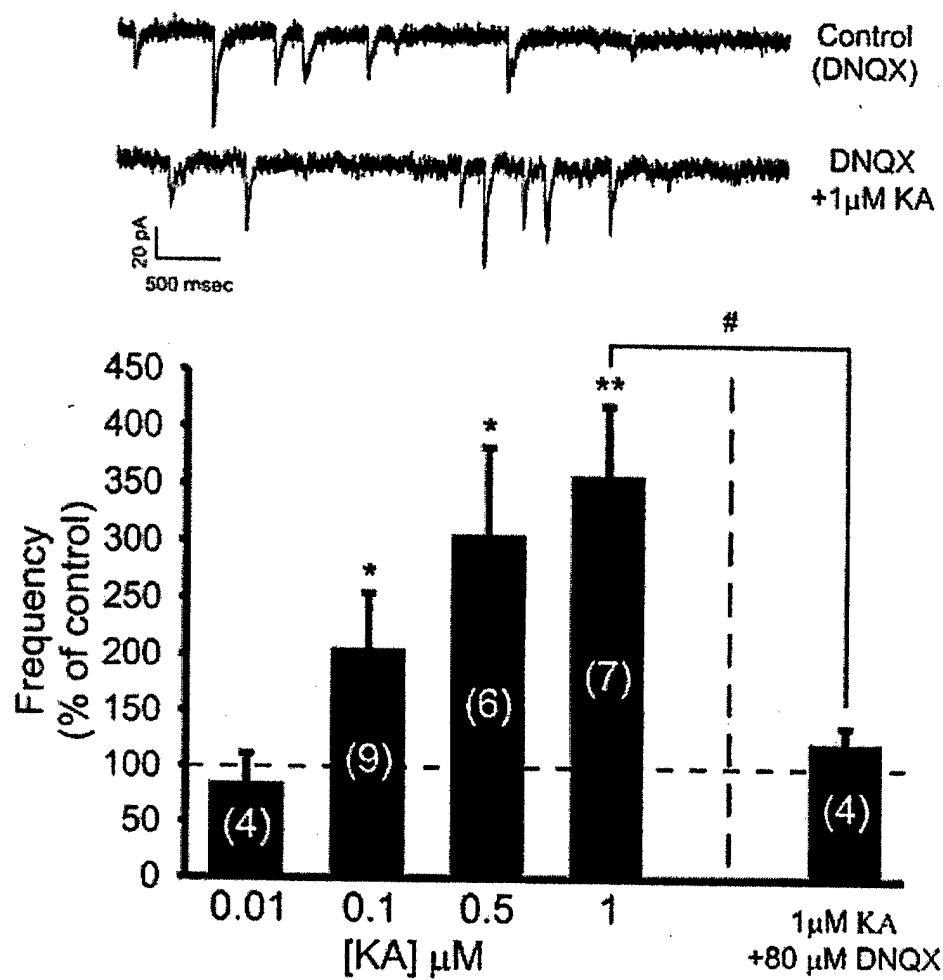
Figure 5: Responsiveness of the postsynaptic neurons in the NAcc to GABA is not altered by KA receptor activation. *A*: Representative traces demonstrating GABA_A receptor-mediated currents elicited in NAcc neurons by direct pressure application of 10 μ M GABA in the absence and presence of 1 μ M KA. GABA-evoked currents are blocked by 20 μ M bicuculline. Traces are averages of four currents evoked by GABA under baseline, KA, wash, and bicuculline conditions. *B*: Summary bar graph comparing the effect of 1 μ M KA on evoked GABA_A IPSCs and on GABA_A currents evoked by local pressure application of 10 μ M GABA. Numbers in parentheses indicate number of cells in each condition; * indicates significant difference from control, $p < 0.05$.

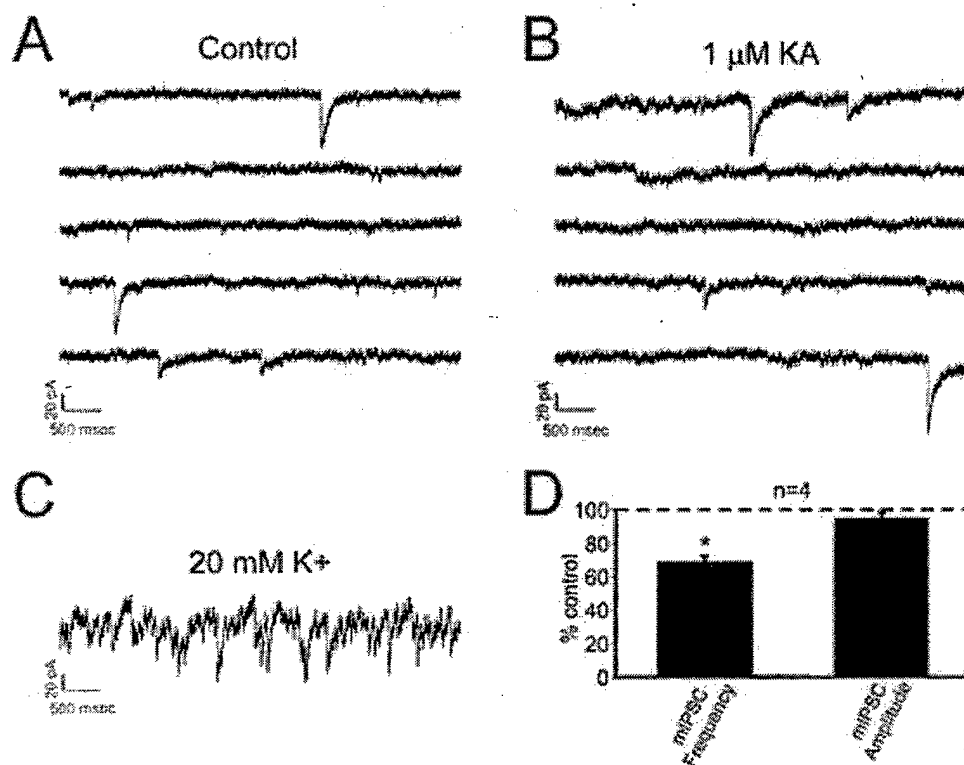
Figure 6: KA effect on GABAergic synaptic transmission in the NAcc is dependent on neuronal excitability. Bar graph showing the effect of 1 μ M KA on eIPSC amplitude in the NAcc in 7 mM Ca^{2+} (High Ca^{2+}) and 0.5 mM Ca^{2+} (Low Ca^{2+}) expressed as percent of control \pm SEM.

Superimposed traces above the graph illustrate the effect of KA on eIPSC amplitude in High Ca^{2+} (left) and Low Ca^{2+} (right). Numbers in parentheses indicate number of cells in each condition; * indicates significant difference from control, $p < 0.05$.

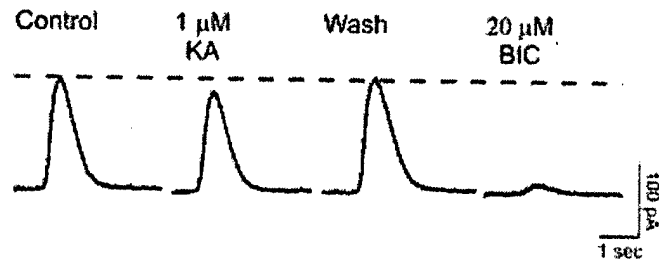




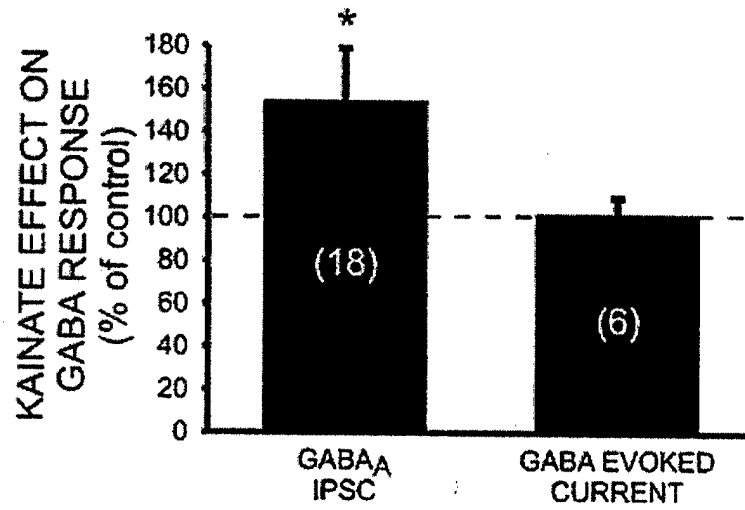


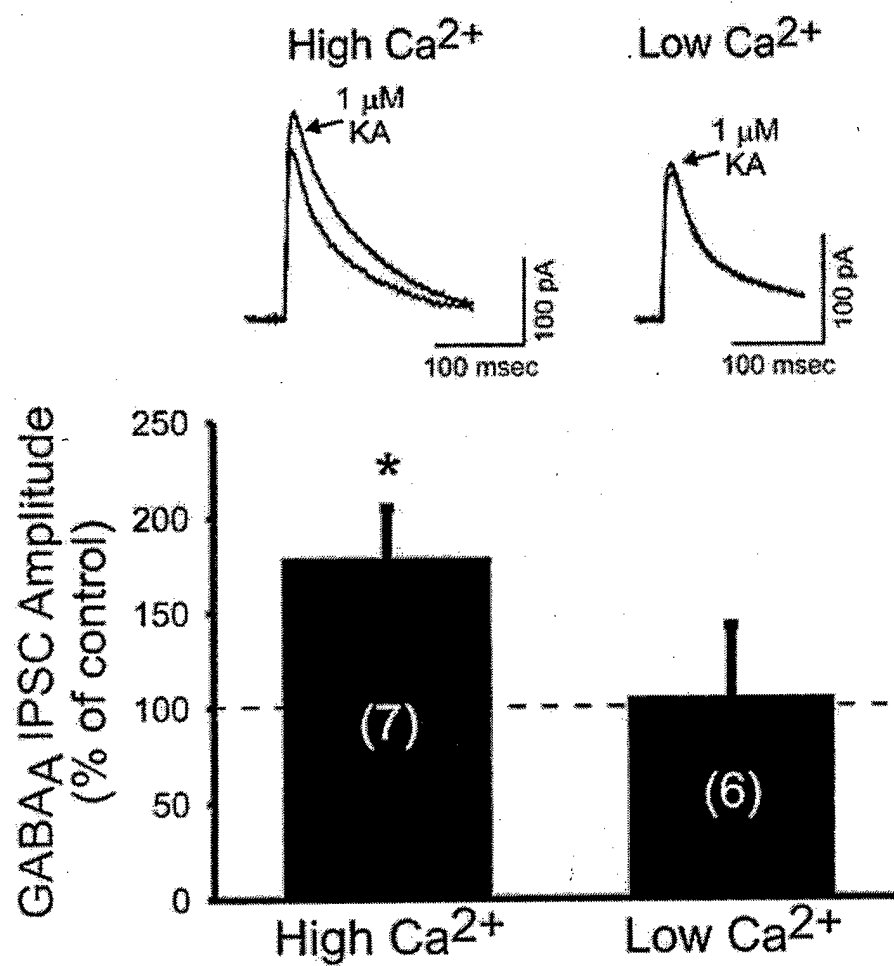


A



B





- Revision Serious -

To: jweiner@wfubmc.edu
From: lwr1@columbia.edu
Date: Aug 12, 2002
CC: lwr1@columbia.edu

Subject: Kainate Receptor Activation Potentiates GABAergic
Synaptic Transmission in the Nucleus Accumbens Core , JN-00509-
2002

Dear Dr. Weiner:

Experts in the field have carefully reviewed your manuscript entitled Kainate Receptor Activation Potentiates GABAergic Synaptic Transmission in the Nucleus Accumbens Core, and, although they found merit in your study, they have raised a number of serious concerns that preclude its acceptance in its present form. Nonetheless, we would be willing to consider a substantially revised resubmission of this manuscript, which will be treated as a new submission. We will endeavor to have the resubmitted manuscript evaluated by two reviewers. We would endeavor to ensure that at least one of the initial reviewers of the manuscript participates in the review of the resubmitted manuscript. The permission to resubmit this manuscript should not be taken to mean that the new submission will necessarily be accepted.

To revise your manuscript, log on to APS Central, <http://www.apscentral.org>, and submit your new manuscript. Please include in your cover letter the information that this is a new submission of a manuscript that was previously rejected with an invitation to submit a thoroughly revised manuscript. Please be sure to include the manuscript number of the present manuscript so that the previous correspondence and reviews are available to the editors. Please include in the cover letter your point by point response to the previous reviewers' comments, and a description of the revisions that you have made.

Thank you very much for submitting your work to the Journal of Neurophysiology.

Sincerely,
Lorna

Dr. Lorna W. Role

Dept. of Cell Biology & Anatomy in the Center for Neurobiology
and Behavior
Columbia University P & S

These are the Editor's comments to the author:

These are the Reviewer's comments to the author:

Referee 1 Comments:

This paper uses slice recordings to test the effect of kainate receptor activation on inhibitory transmission in the rat nucleus accumbens. The results show that exposure to 1 micromolar kainate enhances evoked IPSCs. This dose increases the frequency of spontaneous IPSCs without changing their amplitude, and does not affect miniature IPSC frequency. The study provides new information about the accumbens, but does not break new ground in understanding the mechanisms of kainate receptor mediated modulation.

These are a number of technical issues that need to be cleared up before the study can be considered for publication.

1) Figure 1 uses NBQX to block AMPA receptors, but the evidence for selectivity of this compound is not discussed. The first paragraph of the results should cite Bureau et al. 1999, who first described selective antagonism of AMPA receptors by NBQX in slice recordings, and should also address why the more selective compounds GYKI53655 and SYM2206 were not used in this study.

2) QX-314, a derivative of lidocaine, is typically used to block sodium channels. It is not a classical GABA-B receptor antagonist. The paper should include brief explanation of the basis for GABA-B receptor inhibition by QX-314, with appropriate references to previous studies. The manuscript should emphasize that QX-314 was added to the internal solution, so that it will only influence the recorded cell, GABA-B receptor mediated responses in other cells in the slice should not be affected. The discussion mentions, without showing data, that incubation with conventional GABA-B and adenosine receptor antagonists did not affect the actions of kainate in this system, although modulation of transmission by these receptors has been demonstrated in the accumbens. How might activation of GABA-B receptors have been avoided, given the strong increase in frequency of spontaneous

release? If the spontaneous release illustrated in Fig 2A was insufficient to recruit GABA-B receptors, is it possible to imagine any conditions that would lead to GABA-B activation?

3) Were both calcium and magnesium elevated for the experiments in Fig 6, or only calcium? It was surprising that the change from 7 mM Ca to 0.5 mM Ca caused very little change in the control amplitude of the illustrated IPSCs. Are both the high calcium and low calcium traces from the same cell, or from different cells? The traces for high calcium in Fig 6 also are not representative of the bar graph. The bar shows a mean of approximately 175 \pm 25 % in the presence of kainate, whereas the traces show only about a 120% increase with kainate, which appears to be outside the standard deviation (or is it standard error of the mean?).

4) A previous example in which kainate receptors in presynaptic inhibitory neurons reliably enhanced evoked transmission involved inhibitory synapses onto GABAergic neurons (Cossart et al., 2001; see also Mulle et al., 2000). Is it possible that all of the postsynaptic neurons in the present study were themselves GABAergic? If so, the present report fits well with previous work in the hippocampus. Some discussion of this possibility is needed.

Referee 2 Comments:

The authors investigate the effect of kainate application on evoked, spontaneous and miniature GABAergic signals in NAcc core cells. The main findings are that evoked and miniature IPSCs are reduced, but that spontaneous IPSCs are increased in frequency. The authors argue, mainly on the basis of the discrepancy between effects on miniature and action potential-dependent spontaneous IPSCs, that KA acts on receptors to excite interneurons.

The results are generally clear and most of the reasoning is convincing. However, I am left feeling that this study is incomplete. The authors draw attention to one of the three phenomena and seem to relegate the other two (effects on evoked IPSCs and miniatures) to a lower priority. However, the effects on evoked IPSCs are arguably highly relevant to the transmission of phasic signals through the structure. It would be nice to know why the evoked IPSCs are reduced. One obvious experiment would be

to block presynaptic GABAB receptors, to see whether the increase in spontaneous GABA release causes a presynaptic inhibition

mediated by these receptors, as has been suggested in the hippocampus. If so, it still leaves the question why KA leads to a decrease in mIPSC frequency. Does manipulation of extracellular Ca^{2+} , for instance, interfere with the decrease in mIPSC frequency? These all seem pretty obvious experiments, and I therefore think that it would be premature to terminate the study as it is.

Other specific concerns:

1 How were neurons identified? Were some electrophysiological criteria applied to verify that they were all MSNs?

2 Figure 1 is incompletely documented: was NBQX applied throughout, or just during the KA application? What was the concentration of DNQX? What is the evidence the IPSCs were not contaminated by KA receptor-mediated EPSCs, and that AMPA receptors were fully blocked by 1 μM NBQX? The effect of applying a GABAA receptor blocker should at the very least be shown.

3 Figure 4 and elsewhere: how many cells? What happened with a lower concentration of KA? Was the effect reversible? And what happened if KA was applied in a different concentration of Ca^{2+} ? The data should also be shown as cumulative frequency plots for consistency.

4 Fig 5 B (left) is replotted from Fig 1. This should be pointed out to avoid giving the misleading impression of new data.

5 The results of manipulations of Ca^{2+} are very indirect evidence. Indeed, one could argue that they are opposite to what is expected: reducing neuronal excitability by increasing Ca^{2+} might be expected to decrease the effect of KA! Overall, I think it would be much more convincing to record from interneurons directly, and see what KA application does to them.

6 There is no evidence that endogenous glutamate can do what the

exogenous KA does. This might be feasible by delivering a conditioning train of stimuli.

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Senior Editor: Francis J. White

Alcohol Potently Inhibits the Kainate Receptor-dependent Excitatory Drive of Hippocampal Interneurons

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Keywords: glutamate, ethanol, channel, GABA, hippocampus, acute.

Abstract

Kainate receptors are members of the glutamate-gated family of ionotropic receptors, which also includes NMDA and AMPA receptors. Kainate receptors are important modulators of interneuron excitability in the CA1 region of the hippocampus. Activation of these receptors enhances interneuron firing, which robustly increases spontaneous inhibitory postsynaptic currents in pyramidal neurons. We report here that ethanol (EtOH) potently inhibits this kainate receptor-mediated effect at concentrations as low as those that can be achieved in blood after the ingestion of just 1-2 drinks (5-10 mM). Pressure application of kainate, in the presence of AMPA and NMDA receptor antagonists, evoked depolarizing responses in interneurons that triggered repetitive action potential firing. EtOH potently inhibited these responses to a degree that was sufficient to abolish action potential firing. This effect appears to be specific for kainate receptors as EtOH did not affect action potential firing triggered by AMPA receptor-mediated depolarizing responses. Importantly, EtOH inhibited interneuron action potential firing in response to kainate receptor activation by synaptically-released glutamate, suggesting that our findings are physiologically relevant. Kainate receptor-dependent modulation of glutamate release onto pyramidal neurons was not affected by EtOH. Thus, EtOH increases excitability of pyramidal neurons indirectly by inhibiting the kainate receptor-dependent drive of GABAergic interneurons. We postulate that this effect may explain some of the paradoxical excitatory actions of this widely abused substance. The excitatory actions of EtOH may be perceived as rewarding by some individuals, which could contribute to the development of alcoholism.

Kainate receptors (KA-Rs) are glutamate-gated ion channels that play important roles in the regulation of hippocampal excitability. In mossy fiber-to-CA3 pyramidal neuron synapses, KA-Rs mediate synaptic currents and plasticity and modulate glutamate release presynaptically (Reviewed in (Kullmann, 2001; Schmitz et al., 2001). In the CA1 region, KA-Rs inhibit glutamate release presynaptically (Kamiya and Ozawa, 1998; Vignes et al., 1998; Frerking et al., 2001) and also regulate GABA release from interneurons. Activation of KA-Rs by micromolar concentrations of kainate inhibits monosynaptic GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) in CA1 pyramidal neurons (Clarke et al., 1997; Rodriguez-Moreno et al., 1997; Cossart et al., 1998; Frerking et al., 1998; Min et al., 1999; Crowder et al., In Press). This effect was initially interpreted to indicate that activation of interneuronal KA-Rs exerts a disinhibitory effect on CA1 pyramidal neurons. However, KA-Rs depolarize interneurons and induce repetitive action potential firing, which results in a robust increase in the frequency of GABA_A receptor-mediated spontaneous IPSCs (sIPSCs) recorded in CA1 pyramidal neurons (Cossart et al., 1998; Frerking et al., 1998; Bureau et al., 1999). Importantly, activation of KA-Rs by synaptically-released glutamate enhances axonal excitability in interneurons and increases sIPSC frequency in CA1 pyramidal neurons (Jiang et al., 2001; Semyanov and Kullmann, 2001). Therefore, the KA-R-mediated increase of interneuronal GABA release may function as an important homeostatic mechanism that prevents overexcitation of CA1 pyramidal neurons (Mulle et al., 2000; Jiang et al., 2001; Kullmann, 2001).

KA-Rs have emerged as important potential targets of alcohol's actions in the central nervous system (CNS). Ethanol (EtOH) inhibits recombinant KA-Rs in non-neuronal expression systems and native KA-Rs in cultured neurons at concentrations ≥ 25 mM (legal intoxication limit in the U.S. is 0.08 g/dl = 17 mM) (Dildy-Mayfield and Harris, 1995; Valenzuela et al., 1998; Valenzuela and Cardoso, 1999; Costa et al., 2000). We reported that KA-R-mediated synaptic currents in CA3 pyramidal neurons in hippocampal slices are inhibited by EtOH at concentrations as low as 20 mM (Weiner et al., 1999). Moreover, it was recently discovered that 20-80 mM EtOH inhibits the KA-R-mediated inhibition of monosynaptic IPSCs in CA1 pyramidal neurons (Crowder et al., In Press), suggesting that KA-Rs in interneurons may also be sensitive to pharmacologically-relevant concentrations of this drug of abuse.

In this paper, we provide direct evidence of a potent inhibitory effect of EtOH on interneuronal KA-Rs, which mediate a significant portion of the glutamatergic excitatory drive of these neurons (Cossart et al., 2002). We demonstrate that interneuron firing in response to KA-R activation is inhibited by EtOH concentrations that can be achieved in blood after the ingestion of just 1-2 drinks (5-10 mM). We also show that this effect of EtOH results in a substantial decrease in the frequency of KA-R-driven sIPSCs recorded in pyramidal neurons. We postulate that the effects of EtOH on interneuronal KA-Rs could contribute to some of the paradoxical excitatory actions that can be induced by low doses of this widely abused substance.

Materials and Methods

Unless indicated, all chemicals were from Sigma-RBI-Fluka (St. Louis, MO). Experiments were performed in coronal hippocampal slices that were prepared from 21-40 day-old male Sprague-Dawley rats. For recordings of sIPSC from CA1 pyramidal neurons and all interneuron recordings, rats were anesthetized with ketamine 250 mg/kg and 350-400 μ M thick slices were prepared with a vibratome as described (Shuttleworth and Connor, 2001). Artificial cerebrospinal fluid (ACSF) contained (in mM): 126 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 1 MgSO_4 , 26 NaHCO_3 , 2 CaCl_2 , 0.1 DL-AP5 (Tocris-Cookson, Bristol, U.K.) and 10 glucose, equilibrated with 95% O_2 /5% CO_2 . The AMPA receptor blocker, GYKI 53655 (30 μ M; custom synthesized by Tocris-Cookson), was added to the ACSF to isolate KA-R-mediated responses. When indicated, bicuculline methiodide, DNQX (Alexis Co., San Diego, CA), SHC 50911 (Tocris-Cookson) or EtOH (AAPER Chemical Co., Shelbyville, KY) were added to the ACSF. After a recovery time of ≥ 80 min, slices were transferred to a chamber perfused with ACSF at a rate of 2-3 ml/min. Whole-cell patch-clamp electrophysiological recordings from CA1 pyramidal neurons and stratum radiatum-lacunosum moleculare interneurons were performed under infrared-differential interference contrast microscopy at 34°C with an Axopatch 200B amplifier (Axon Laboratories, Union City, CA). Microelectrodes had resistances of 3-5 $\text{M}\Omega$. We recorded sIPSC at a holding potential of -60 mV using internal solution containing (in mM): 140 CsCl, 2 MgCl_2 , 1 CaCl_2 , 10 EGTA, 10 HEPES (pH 7.3), 2 $\text{Na}_2\text{-ATP}$ and 2 QX-314 (Tocris-Cookson). The effect of KA-R activation on sIPSC frequency ran down after sequential applications of kainate. Therefore, the effect of EtOH on the KA-R-mediated increase of sIPSC frequency was assessed on

neurons from different slices that had not been previously exposed to kainate. Current clamp experiments ($I_{\text{holding}} = 0$ pA) were performed with an internal solution containing (in mM): 135 K-gluconate, 10 MgCl_2 , 0.1 CaCl_2 , 1 EGTA, 10 HEPES (pH 7.3), 2 $\text{Na}_2\text{-ATP}$. Input resistances were between 20-35 M Ω . A pneumatic picopump (World Precision Instruments, Sarasota, FL) was used to apply puffs of kainate or AMPA (Tocris-Cookson). The puffing pipette was placed ~ 200 μm from the cell, the pressure was varied between 4-7 psi and the duration was between 2-10 sec. EPSPs were evoked with a concentric bipolar electrode (inner pole, 25 μm ; outer pole, 125 μm ; FHC, Bowdoinham, ME) placed in the stratum radiatum. Data were acquired and analyzed with pClamp7 or 8 (Axon Laboratories); sIPSCs were analyzed with Minis Analysis program (Synaptosoft, Decatur, GA). For the experiments on the presynaptic actions of kainate on glutamatergic terminals, slice preparation and recording methods were as described (Weiner et al., 1999). Data are presented as mean \pm S.E.M. Statistical analyses were performed with Prism (GraphPad Software, San Diego, CA).

Results

To initially assess the effect of ethanol on interneuronal KA-R function, we studied the modulation by kainate of GABAergic tone in CA1 pyramidal neurons in rat hippocampal slices. We recorded IPSCs evoked in pyramidal neurons by the spontaneous release of GABA from interneurons; whole-cell voltage-clamp recordings were performed in the presence of GYKI 53655 (30 μ M) and DL-AP5 (100 μ M) to block AMPA and NMDA receptors, respectively. Spontaneous IPSCs were blocked by bicuculline methiodide, indicating that they were mediated by GABA_A receptors (Fig 1A; similar results seen in 2 additional neurons). Application of 0.5 μ M kainate induced a robust and reversible increase in sIPSC frequency (Fig 1B-C; Fig 2). Ethanol potently and reversibly inhibited this effect of kainate (Fig 1D-E; Fig 2). The KA-R-mediated increase in sIPSC frequency was blocked by the non-NMDA receptor antagonist, DNQX (Fig 1F-G; similar results seen in 3 additional neurons). Since GYKI 53655 was present during these experiments, this finding confirms that the effect of kainate was mediated by KA-Rs. Non-linear regression analysis yielded an EtOH IC₅₀ of 4.4 mM (confidence interval 1.6-11.6 mM; Fig 2, inset). Application of EtOH (2-25 mM) alone did not significantly affect basal sIPSC frequency (Fig 1D-E; Fig 2); 50 mM EtOH induced a small but significant increase in sIPSC frequency (Fig 2). The average sIPSC amplitudes in the presence of 2, 5, 10, 25, and 50 mM EtOH were 4.9 ± 3.7 (n = 10), 1.9 ± 3.2 (n = 8), 3.1 ± 3.5 (n = 7), -0.5 ± 6.9 (n = 7), and 0.9 ± 2.6 (n = 9) % of control, respectively (data not shown; see Fig 1D-E for trace illustrating the lack of an effect of 10 mM EtOH).

To further assess the effect of ethanol on KA-R function, we recorded directly from interneurons under whole-cell current-clamp conditions. We studied interneurons located in the stratum radiatum near the stratum lacunosum moleculare. As shown in Fig 3, pressure application of kainate (5 μ M in the micropipette located \sim 200 μ m from the patched interneuron) in the presence of GYKI 53655 (30 μ M) and DL-AP5 (100 μ M), caused a reversible depolarization (14 ± 2 mV; $n = 8$) and repetitive action potential firing (42 ± 7 action potentials per evoked response; $n = 8$) in these neurons. Pressure application of the same AMPA concentration in the absence of GYKI, reversibly depolarized the interneurons to a similar extent (21 ± 3 mV; $n = 7$) and also caused them to fire repetitive action potentials (95 ± 15 action potentials per evoked response; $n = 7$). Bath application of EtOH (10 mM) significantly reduced the amplitude of KA-R-mediated evoked potentials sufficiently to abolish action potential firing (Fig 3). In contrast, EtOH did not significantly affect the amplitude of AMPA receptor-mediated evoked potentials or the action potential firing in response to AMPA (Fig 3). Application of EtOH alone did not significantly affect the interneuronal resting membrane potential (control = -68 ± 3 mV and EtOH = -70 ± 3 mV; $n = 15$).

We next assessed the effect of EtOH on KA-R-mediated interneuron EPSPs evoked by synaptic glutamate release from Schaffer collateral axonal terminals. These experiments were also performed in the whole-cell current clamp mode. EPSPs were evoked by trains of 5 stimuli at 20 Hz to maximize the induction of action potential firing. In the presence of blockers of NMDA (DL-AP5; 100 μ M), GABA_A (bicuculline methiodide; 25 μ M) and GABA_B (SCH-50911; 20 μ M) receptors, repetitive stimulation of

the Schaffer collateral reproducibly induced non-NMDA receptor-mediated EPSPs (peak amplitude: 29 ± 2 mV; $n = 4$) that triggered action potential firing. In agreement with a recent report (Frerking and Ohliger-Frerking, 2002), GYKI 53655 (30 μ M) reduced the peak amplitude of the first non-NMDA EPSP by 63 ± 5 % ($n = 4$) and abolished action potential firing (Fig 4A-C). In the continuous presence of GYKI, an increase in stimulation intensity enhanced EPSP amplitude and restored action potential firing (Fig 4A-C). A subsequent application of EtOH (10 mM) significantly reduced the amplitude of the KA-R-mediated EPSPs and reduced action potential firing (Fig 4A-D). These events were abolished by DNQX (Fig 4A-C). It should be noted that paired-pulse facilitation of KA-R-mediated EPSPs was not affected by EtOH; the ratio of the amplitude of the first to the second EPSP were 2 ± 0.4 ($n = 4$) and 2.5 ± 0.8 ($n = 4$) in the absence and presence of EtOH, respectively.

Finally, we determined the effect of EtOH on presynaptic KA-R-dependent modulation of glutamate release in Schaffer collateral-to-CA1 pyramidal neuron synapses. For these experiments, we recorded AMPA receptor-mediated EPSCs that were evoked by electrical stimulation of the stratum radiatum in the presence of D-AP5 (50 μ M) and bicuculline methiodide (20 μ M). In agreement with previous reports (Kamiya and Ozawa, 1998; Vignes et al., 1998; Frerking et al., 2001), we found that 1 μ M kainate inhibited the amplitude of AMPA EPSCs (Fig 5A,C). EtOH (40 and 80 mM) alone had no effect on the amplitude of AMPA EPSCs and it did not significantly affect the kainate-induced inhibition of AMPA receptor-mediated EPSCs (Fig 5B,C). A comparable result was obtained for NMDA receptor-mediated EPSCs recorded in the presence of

bicuculline methiodide (20 μ M) and NBQX (1 μ M). NMDA receptor-dependent EPSCs were inhibited by $46 \pm 4\%$ ($n = 17$) and $53 \pm 4\%$ ($n = 11$) by 1 μ M kainate in the absence and presence of 80 mM EtOH, respectively (data not shown).

Discussion

We demonstrate here that EtOH potently inhibits KA-R function in CA1 hippocampal interneurons. We initially tested the effect of EtOH on KA-R-mediated modulation of GABAergic tone in this region. In agreement with previous reports, we found that bath application of kainate, in the presence of AMPA and NMDA receptor blockers, enhances GABA release from interneurons, resulting in a massive increase in sIPSC frequency in pyramidal neurons (Cossart et al., 1998; Frerking et al., 1998; Mulle et al., 2000). Importantly, we found that EtOH inhibits this KA-R-dependent effect with an IC_{50} of 4.4 mM. By comparison, a number of studies have reported that EtOH concentrations ≥ 10 mM are required to significantly inhibit NMDA receptor-dependent population EPSPs in the CA1 region of hippocampal slices (Lovinger et al., 1990; Morrisett et al., 1991; Swartzwelder et al., 1995). Moreover, significant inhibition of AMPA receptor-mediated population EPSPs was only observed with 100 mM EtOH in this hippocampal region (Lovinger et al., 1990). This result is in agreement with our finding that AMPA receptor-mediated responses in CA1 pyramidal neurons and interneurons are not significantly affected by EtOH. Consequently, interneuronal KA-Rs appear to be the most EtOH sensitive glutamatergic ionotropic receptor subtype in the CA1 region of the adult rat hippocampus. This conclusion can be extended to the CA3 hippocampal region, where we found that KA-Rs are the only members of the ionotropic glutamate receptor family that are significantly inhibited by 20-40 mM EtOH (Weiner et al., 1999).

Our study also demonstrates that 2-50 mM EtOH does not affect the amplitude of basal GABA_A-mediated sIPSCs. This result is in general agreement with several reports that concentrations of EtOH \geq 40 mM are required to significantly potentiate GABA_A receptor-mediated postsynaptic responses in CA1 pyramidal neurons (Weiner et al., 1997a; Weiner et al., 1997b; reviewed in Little, 1999). Moreover, we found that EtOH, at concentrations \leq 25 mM, does not significantly affect basal sIPSC frequency, indicating that it does not directly modulate GABA release in response to spontaneous action potential generation in CA1 interneurons. It also suggests that spontaneous GABAergic transmission is not under the tonic control of KA-Rs under our recording conditions. If this had been the case, we would have expected to observe inhibition of basal sIPSC frequency in the presence of EtOH. This finding is not completely unexpected given that glutamate is just one of the many neurotransmitters that regulate spontaneous firing in CA1 hippocampal interneurons (Parra et al., 1998). Indeed several studies have demonstrated that blockade of glutamate receptors does not significantly inhibit basal sIPSC frequency in hippocampal pyramidal neurons (McBain et al., 1992; Bergles et al., 1996). It should be noted, however, that KA-Rs may control basal spontaneous action potential generation in CA1 interneurons under some conditions; it was recently established that KA-R-mediated spontaneous and miniature EPSCs can contribute ~50% of the glutamate-induced current in CA1 interneurons in slices from immature rats (Cossart et al., 2002).

Crowder *et al* (Crowder et al., In Press) recently found that EtOH, at concentrations \geq 20 mM, significantly inhibits the kainate-induced inhibition of monosynaptic IPSCs in CA1

pyramidal neurons. In contrast, the results of the present study indicate that EtOH concentrations as low as 5 mM are sufficient to inhibit the KA-R-mediated increase in sIPSC frequency in these neurons. This discrepancy could be due to the fact that a higher concentration of kainate (1 μ M) was used in the study of Crowder *et al* (Crowder *et al.*, In Press) because it has been shown that the effect of EtOH on KA-Rs can be inversely related to the concentration of agonist used to activate the receptor (Valenzuela and Cardoso, 1999). Moreover, studies by Lerma and collaborators have suggested that two different populations of KA-Rs may mediate the inhibition of monosynaptic IPSCs and the potentiation of sIPSC frequency (Rodriguez-Moreno and Lerma, 1998). For instance, the kainate-induced inhibition of monosynaptic IPSCs, but not the potentiation of sIPSCs, has been shown to depend on the activation of a metabotropic cascade involving $G_{i/o}$ proteins, phospholipase C, and protein kinase C in the presynaptic terminal (Rodriguez-Moreno and Lerma, 1998). In addition, low concentrations of glutamate cause a significant reduction in monosynaptic IPSCs amplitude without affecting sIPSC frequency (Rodriguez-Moreno and Lerma, 1998). Thus, it is possible that KA-Rs in presynaptic terminals have lower sensitivity to EtOH than those in somatodendritic and/or axonal compartments, which could be due to differences in the subunit composition or post-translational regulation of these receptors.

A key finding of our study is that the mechanism of action of EtOH involves an all-or-none effect on interneuronal firing. We found that EtOH inhibited KA-R-mediated evoked potentials to a degree that is sufficient to abolish interneuron firing in response.

to activation of these receptors. This finding explains the relatively steep relationship between EtOH concentration and inhibition of the kainate-induced increase in sIPSC frequency. This steep concentration dependence likely reflects the EtOH-induced progressive inhibition of KA-R-evoked firing in the different interneurons that synapse onto the particular pyramidal neuron under study. Interestingly, 10 mM EtOH inhibited responses evoked by pressure application of kainate by only ~25%; however, the kainate-induced increase in sIPSC frequency was reduced by ~60% by this concentration of EtOH. Given that we likely pressure-delivered concentrations of kainate $\leq 0.5 \mu\text{M}$, our findings suggest that a relatively modest effect of ethanol on interneuronal KA-Rs becomes amplified into a more substantial decrease in tonic inhibition of principal neurons. This suggestion is important given that pharmacologically relevant concentrations of EtOH have generally been found to produce relatively small effects on the function of neurotransmitter-gated and voltage-gated ion channels (For a review, see (Little, 1999)). Thus, our results clearly illustrate that effects of EtOH, which may seem minimal when examined in isolated neurons, could have a dramatic impact on the excitability of neurons when studied in the context of a circuit such as the one studied here.

Our study also shows that EtOH inhibits interneuron firing in response to KA-R activation by synaptically released glutamate. This finding suggests that the results obtained with exogenous application of kainate have physiological relevance.

We postulate that the effects of EtOH on interneuronal KA-Rs are likely to have profound effects on the excitability of pyramidal neurons as illustrated by the simplified

model shown in Fig 6. Since interneuronal AMPA receptors are insensitive to EtOH, these receptors have been removed from the model for clarity. It should be kept in mind, however, that these receptors mediate a significant portion of the excitatory drive onto interneurons. Our model assumes that glutamate is being actively released onto interneurons, which activates somatodendritic (and/or axonal; not shown) KA-Rs and depolarizes interneurons. Depolarization induces action potential firing, which in turn increases tonic GABAergic inhibition of pyramidal neurons. In the presence of low concentrations of EtOH, interneuronal KA-Rs are inhibited and firing is reduced, leading to a decrease in GABA release from interneurons. EtOH, at concentrations ≤ 10 mM, is not expected to significantly affect the function of most postsynaptic receptors and ion channels in CA1 or CA3 pyramidal neurons. Moreover, we demonstrated that the function of presynaptic KA-Rs at glutamatergic terminals is not affected by EtOH concentrations as high as 80 mM. Therefore, the net effect of 5-10 mM EtOH should be to increase the excitability of CA1 pyramidal neurons. In contrast, higher EtOH concentrations would likely reduce hippocampal excitability by inhibiting NMDA receptors and potentiating GABA_A receptors in CA1 pyramidal neurons, and KA-Rs in CA3 pyramidal neurons. Presumably, these effects would overshadow the excitatory effect produced by inhibition of interneuronal KA-Rs and cause overall depression of pyramidal neurons.

Numerous studies have established that at concentrations produced by the ingestion of 1-2 drinks (5-10 mM), EtOH produces paradoxical excitatory effects, including memory facilitation (Parker et al., 1981; Phillips and Shen, 1996; Rossetti et al., 2002).

Importantly, an increase in the spontaneous firing rate of single units in the dorsal hippocampus has been demonstrated after injection of low doses of EtOH into rats (Grupp, 1980). However, mechanisms explaining how a powerful CNS depressant such as EtOH can produce these paradoxical excitatory effects have remained elusive. The results of our study suggest that inhibition of the KA-R-dependent excitatory drive of inhibitory interneurons could explain, at least in part, some of these stimulatory actions of EtOH, which may be perceived as rewarding by some individuals and contribute to the development of alcoholism.

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Figure Legends

Fig 1. EtOH inhibits the KA-R-mediated increase of sIPSCs frequency in CA1 pyramidal neurons. (A) sIPSCs are fully blocked by the GABA_A receptor antagonist, bicuculline (BIC; 25 μ M). (B-C) Bath application of kainate (0.5 μ M) for 3.5 min, in the presence of GYKI 53665 (30 μ M) and DL-AP5 (100 μ M), induced a robust and reversible sIPSC frequency increase. (D-E) EtOH (10 mM) did not affect the basal amplitude and frequency of sIPSCs but reduced the increase in frequency induced by KA-R-activation. (F-G) In the presence of DNQX (80 μ M), kainate failed to increase sIPSC frequency. Scale bars: 100 pA and 250 ms.

Fig 2. EtOH inhibits the KA-mediated effect on sIPSC frequency in a dose-dependent manner.

The solid bar represents the average percent change induced by kainate (0.5 μ M) on sIPSC frequency. Striped bars represent the effect of increasing concentrations of EtOH on this KA-R-dependent effect. Open bars represent the percent change in basal sIPSC frequency induced by EtOH alone. The inset shows a non-linear regression fit of the inhibitory effect of EtOH on the KA-R-induced increase of sIPSC frequency ($IC_{50} = 4.4$ mM). Each bar or symbol represent the mean \pm SEM of 7-28 neurons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by one-way ANOVA followed by Bonferroni's multiple comparison test; # $p < 0.05$, by one sample *t*-test versus theoretical mean of zero.

Fig 3. EtOH inhibits evoked potentials and action potential firing triggered by pressure application of kainate.

(A) Sample traces of current clamp recordings ($I=0$) from CA1 stratum radiatum-stratum lucidum interneurons. *Upper traces*: in the presence of DL-AP5 (100 μ M) and GYKI 53655 (30 μ M), pressure application of kainate (5 μ M in micropipette located \sim 200 μ m from the soma) induced reproducible evoked potentials and bursts of action potential firing. Bath application of EtOH (10 mM) induced a reversible decrease of the KA-R-mediated evoked potentials and also abolished firing. *Lower traces*: same experiment was performed by pressure-delivering AMPA instead of kainate in the presence of DL-AP5 only. EtOH did not inhibit AMPA receptor-mediated evoked potential amplitude and action potential firing. Scale bars: 5 mV and 5 sec. (B) Summary of the effects of EtOH on KA-R-dependent and AMPA receptor-dependent evoked potential amplitude and action potential number. Each bar represents the mean \pm SEM of 7-8 neurons. ** $p < 0.01$, *** $p < 0.001$, by one sample t -test versus theoretical mean of 100.

Fig 4. EtOH inhibits KA-R-mediated EPSP and action potential firing evoked by stimulation of the Schaffer collateral.

(A) Sample traces of current clamp recordings ($I=0$) from a CA1 stratum radiatum-stratum lucidum interneuron. (a) In the presence of DL-AP5 (100 μ M), bicuculline (25 μ M), and SCH 50911 (20 μ M), a train of five stimuli (20 Hz) delivered in the stratum radiatum induced a non-NMDA EPSP that triggered a burst of action potentials. (b) GYKI 53665 (30 μ M) blocked the AMPA component of the EPSP and eliminated firing. (c) An elevation in the stimulation intensity increased the amplitude of the KA-R-

mediated EPSP and restored action potential firing. (d-e) Bath application of EtOH (10 mM) reversibly decreased the amplitude of the KA-R-mediated EPSP and reduced action potential number. (f) The KA-R-mediated EPSP was fully blocked by DNQX (80 μ M). Scale bars: 5 mV and 5 sec. (B and C) Time-courses illustrating the effects of the sequential application of GYKI, EtOH and DNQX on EPSP amplitude and action potential number in the same cell illustrated in panel A. (D) Summary of the effect of EtOH (10 mM) on action potential (AP) number and EPSP amplitude. Each bar represents the mean \pm SEM of 5 neurons. * $p < 0.05$, ** $p < 0.01$, by one sample t-test versus theoretical mean of 100.

Fig 5. EtOH has no effect on presynaptic kainate receptor-dependent inhibition of AMPA EPSCs in rat hippocampal CA1 pyramidal neurons. Traces are averages of 5-9 AMPA EPSCs recorded in the presence of bicuculline (20 μ M) and D-AP5 (50 μ M). Representative traces illustrating the inhibitory effect of 1 μ M kainate on AMPA receptor-mediated EPSCs in the absence (A) and presence (B) of 80 mM EtOH. Note that EtOH alone has no effect on the amplitude of AMPA EPSCs or kainate inhibition of AMPA EPSCs. (C) Summary of the effect of EtOH on kainate inhibition of AMPA EPSCs. ***, $p < 0.001$ with respect to control by one-way ANOVA; each bar represents the mean \pm SEM of 7-27 neurons.

Fig 6. Simplified model of the EtOH-induced inhibition of the KA-R-mediated excitatory drive of interneurons. This model is based on that proposed by Ben-Ari and collaborators (Cossart et al., 1998). For discussion of other models, see text.

AMPA receptors and axonal KA-Rs are not depicted for clarity. **Left Panel**, under control conditions, glutamate (circles) released from the Schaffer collateral (SC) activates interneuronal KA-Rs, which depolarizes interneurons (I) and induces action potential firing. This effect massively increases GABA (triangles) release onto CA1 pyramidal neurons (P). Presynaptic KA-Rs in Schaffer collateral glutamatergic terminals that synapse onto CA1 pyramidal neurons are depicted in grey. Activation of these receptors has been shown to inhibit glutamate release. The net effect of KA-Rs activation is to increase tonic inhibition of the CA1 pyramidal neurons excitability. **Right Panel**, in the presence of low concentrations of EtOH, KA-Rs in interneurons are inhibited, which reduces the excitatory drive to these neurons, reduces GABA release and increases excitability of pyramidal neurons. KA-Rs in glutamatergic terminals are unaffected by EtOH. Higher concentrations of EtOH would be expected to reduce pyramidal neuron excitability via inhibition of NMDA receptors and/or potentiation of GABA_A receptors at the postsynaptic level.

Fig. 1 (Carta et al)

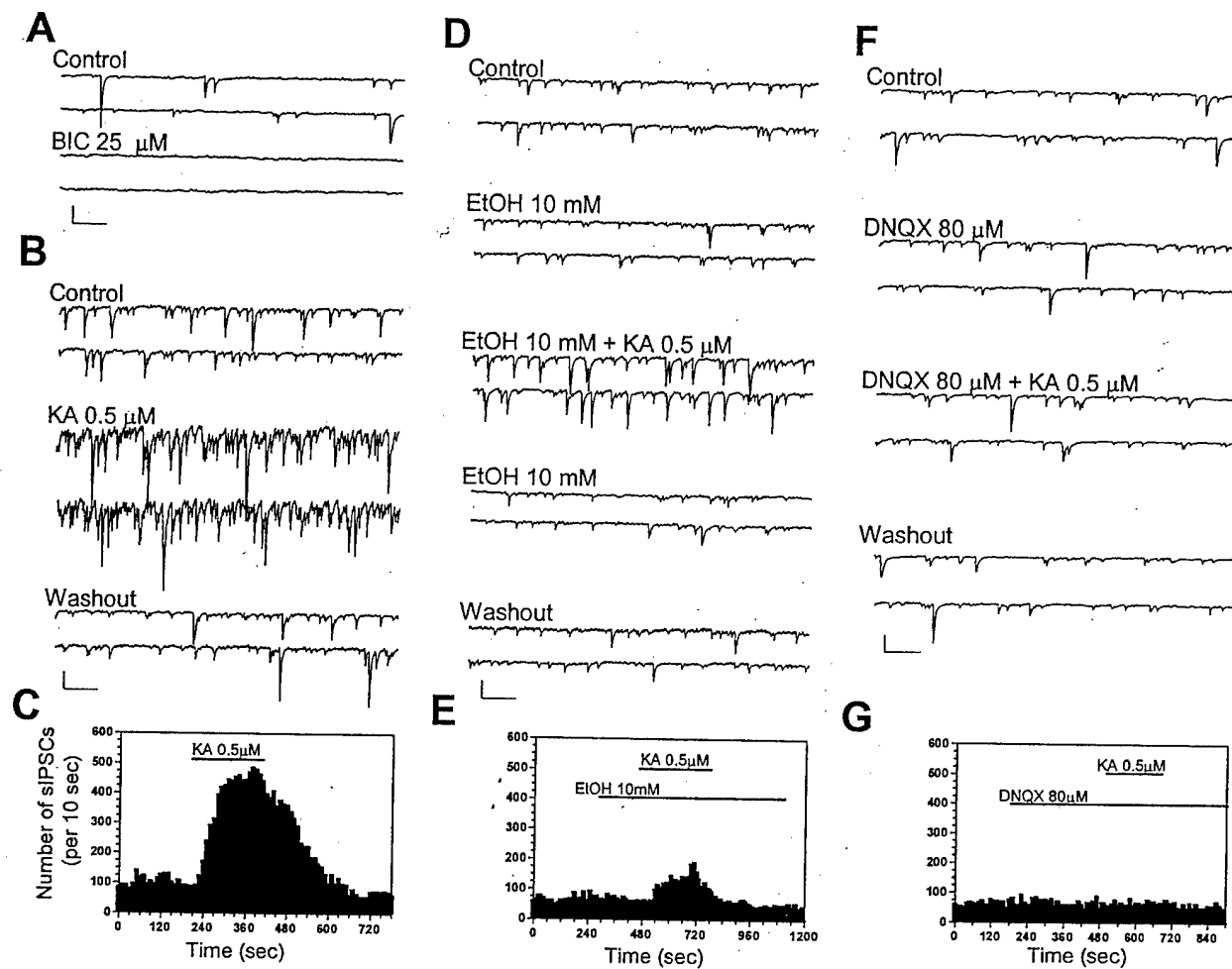


Fig. 2 (Carta et al)

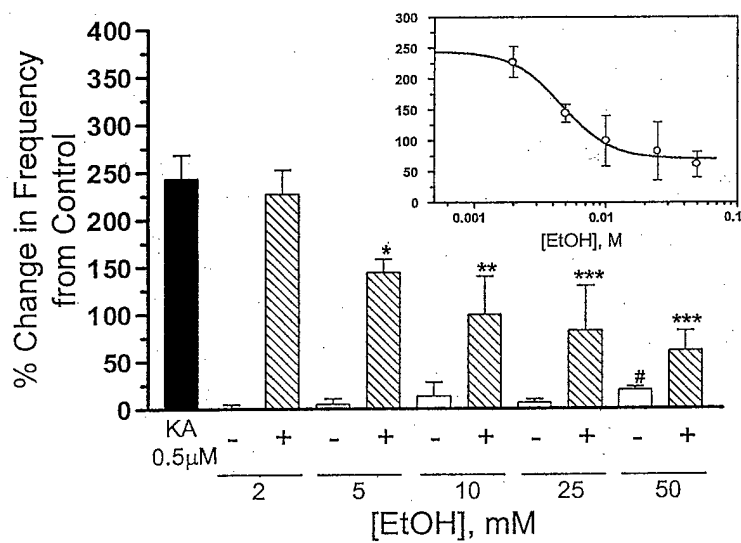


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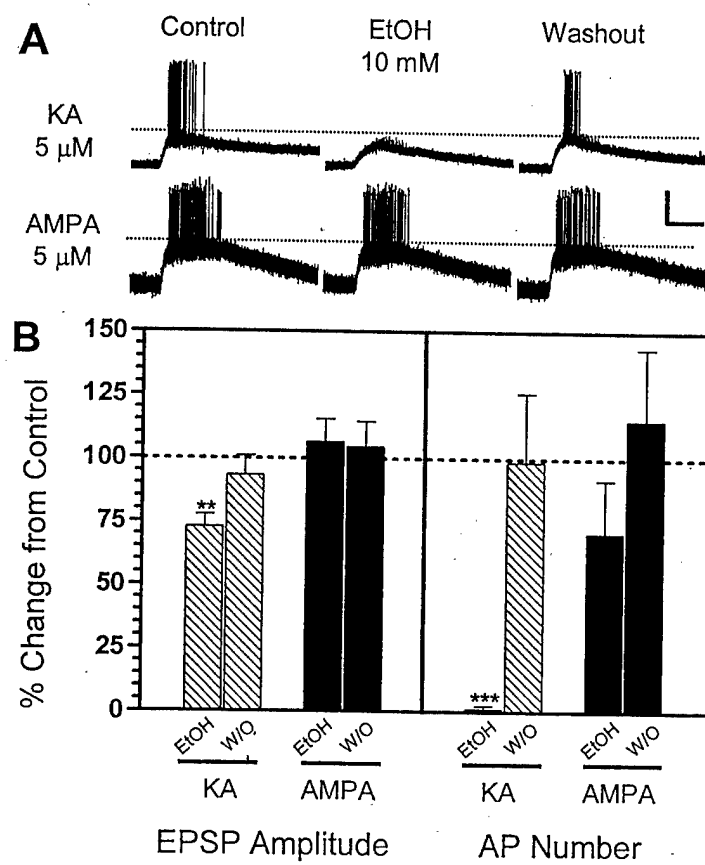


Fig. 4 (Carta et al)

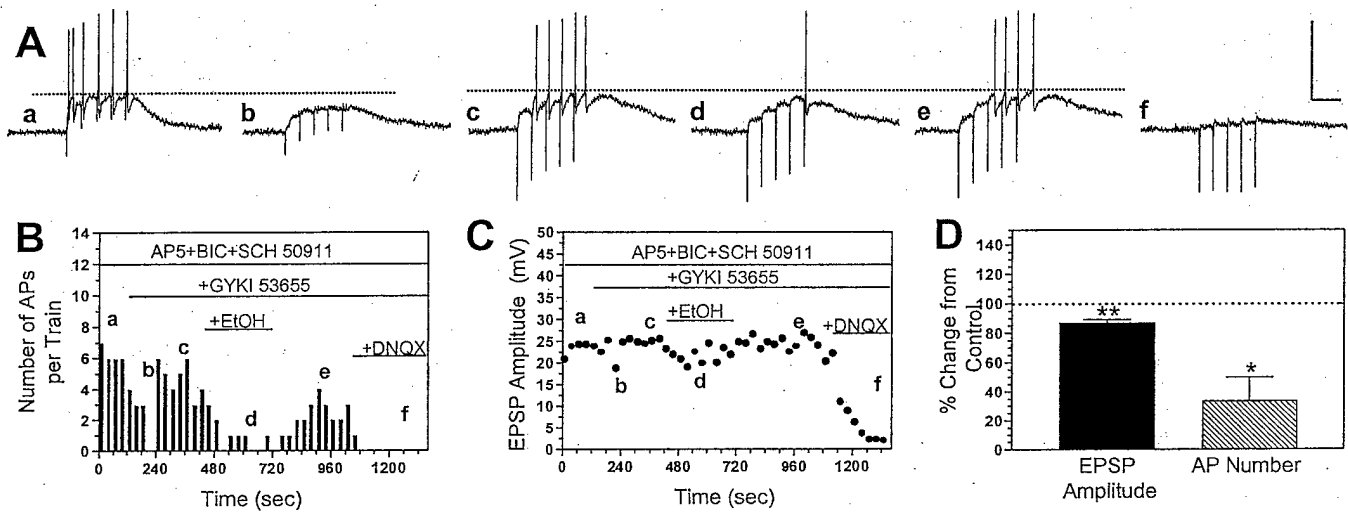


Fig. 5 (Carta et al)

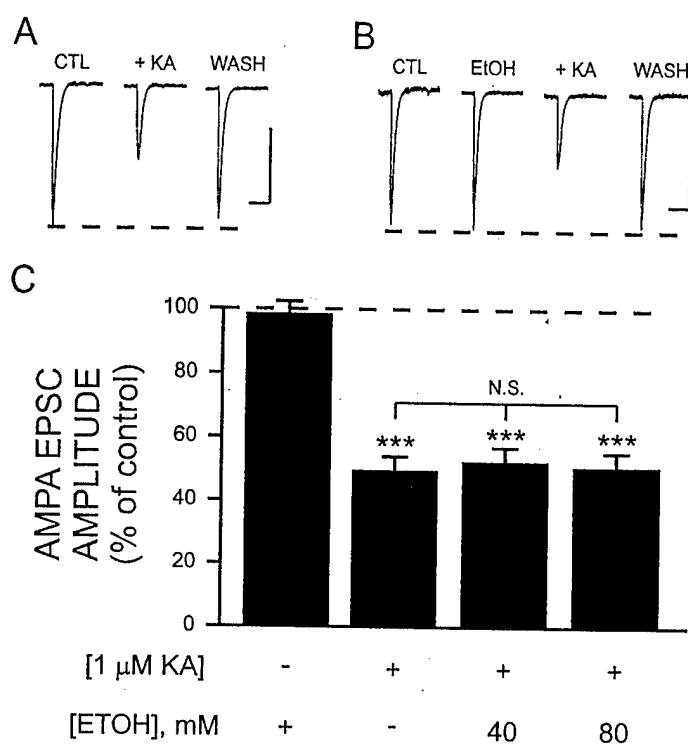


Fig. 6 (Carta et al)

